Translational Biomarker Research for Militarily Relevant Populations in Neurocognitive Diseases

Thesis

Translational Biomarker Research for Militarily Relevant Populations in Neurocognitive Diseases

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A thesis submitted for the degree of Doctor of Philosophy
in the discipline of Neuroscience

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Date of Submission: October 2016

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Declaration

I hereby declare that the work presented in this thesis is my own, except where stated. This work has not been submitted for any other degree or professional qualification.

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Acknowledgements

I would like to extend my deepest thanks to my supervisors Dr. Gogce Crynen and Dr. Fiona Crawford for their advice, guidance, support and involvement throughout the course of my Ph.D. They have given me the opportunity for this research apprenticeship and provided the funding to conduct the experiments detailed in this thesis.

My sincerest gratitude and appreciation goes to Dr. Laila Abdullah and Jon Mike Reed for their friendship, wise advice and countless hours of practical teaching and assistance regarding lipidomics and proteomics experiments and theory, respectively. I would also express my immense gratitude to Dr. Ghania Ait-Ghezala, James Evans and Dr. Michael Mullan for their advice and work discussions.

I would like to thank Ariel Gonzalez for his inspiring friendship and help with genetic studies, as well as Dr. Gary Laco and Thinh Nguyen for their assistance in lipidomic data extraction. I would also like to express my thanks to Dr. Benoit Mouzon and Dr. Joseph Ojo for providing animal samples for my omics experiments.

Special thank you goes out to our external collaborators Major Michael Dretsch, Nancy Klimas and Kimberly Sullivan, who have provided clinical samples for research projects in Traumatic Brain Injury, Posttraumatic Stress Disorder and Gulf War Illness.

I would also like to thank my fellow friends and PhD Students Ben Shakleton, Moustafa Algamal, Cillian Lynch, Utsav Yoshi, Alexander Morin, Jonas Schweig as well as Zuchra Zakirova, Megha Verma and Robert Pelot for their support through this entire process.

I would like to thank my family and my fiancé Justin Martin for their unwavering support, love and believe in me. They have encouraged me in all of my pursuits and inspired me to follow my dreams. This journey would not have been possible without them.

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Posters

- Emmerich T., Abdullah L., Evans J., Reed J., Crynen G., Bishop A., Hart A., Chaytow H., Mouzon B., Ferguson S., Montague H., Pelot R., Gonzalez A., Mullan M., Crawford F., Dretsch M. *Deployment related biomarker discovery in an active military population using an Omics platform*. Poster session presented at: 43nd Annual Society for Neuroscience (SfN) meeting; 2013 November 11; San Diego, CA.
- Emmerich T., Crynen G., Reed J., Abdullah L., Evans J., Crawford F. *Identification of plasma biomarkers of Gulf War Illness using omic technology.* Poster session presented at VA Research Day, James A. Haley Veterans' Administration Hospital; 2014 June 19; Tampa, FL.
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- Abdullah L., Evans J., Emmerich T, Nguyen T., Howland M., Crynen G., Reed J., Shackleton B., Mullan M., Crawford F., Bachmeier C. *Lipidomic studies identify imbalance of omega-6 and omega-3 fatty acid containing phospholipids in blood as early markers of Alzheimer's disease.* Poster session presented at: Kern Lipid Conference; 2015 August 3; Vail, CO.

Abstract

In recent decades more soldiers are being mobilized to conflict areas, such as the over 2 million service members, who have been deployed to Iraq and Afghanistan since October 2001, which includes but is not limited to Operation Enduring Freedom (OEF) and Operation Iraqi Freedom (OIF); or the 700,000 service veterans deployed to the Persian Gulf War in 1990-91 in the US. The UK mobilized over 46,000 military personnel to Iraq, 9,500 British troops to Afghanistan and 50,000 troops to the Gulf War. Soldiers are being exposed to traumatic events such as physical and psychological trauma, as well as chemical exposure and therefore service members are at risk of post-deployment health-related issues, associated commonly with post-traumatic stress disorder (PTSD) and traumatic brain injury (TBI) among OEF/OIF veterans, as well as Gulf War Illness (GWI) among the Persian Gulf War Veteran population.

Although progress has been made in identifying underlying pathology for TBI and PTSD and acute as well as sub-acute biomarkers have been identified, with commercially available tests on the horizon, the work presented here addresses a critical but under-investigated issue, the need for chronic biomarkers for these conditions, as they can go undetected for an extended period of time. Additionally, more evidence has surfaced that discusses how symptoms related to mild TBI (mTBI) can last for years after the insult, emphasizing the importance of investigatjng biomarkers at a late timepoint after injury as, owing to the mild nature of the injury, the condition was often undiagnosed at the time. PTSD itself still lacks an objective measure that can capture its complexity, whereas co-morbidity of PTSD with TBI further complicates the issue. The other mentioned militarily relevant condition, termed GWI, faces similar issues. Veterans

deployed to the Persian Gulf War in 1991 suffer from a disease that has shown to exhibit persistent multisymptom complexity. No biomarker has been identified for this particular population thus making objective diagnosis difficult.

Besides the identification of clinical biomarkers, much research has been done in preclinical models, yet there is still a need to verify and validate such animal models in order to demonstrate their utility. Once the validity of a preclinical model has been confirmed, investigation of pathogenic mechanisms in those models has the potential to reveal therapeutic targets of relevance to the human condition.

Chapter 1 will discuss epidemiology, current clinical diagnosis and pathophysiology of TBI, PTSD and GWI as well as the status of biomarker research in each of these three areas. The thesis then focuses on the identification of plasma biomarkers in human patient populations, specifically in military populations suffering from TBI, PTSD or both at chronic time points post traumatic exposure (Chapters 2 & 3). In Chapter 4, we then explore whether or not such changes are present in our established animal model of TBI. In Chapter 5 we investigate peripheral biomarkers in plasma samples from Gulf War veterans and in two animal models of GWI. Given the complexity of TBI, PTSD and GWI clinical presentation and pathogenesis and their heterogeneity in human populations, it is anticipated that a valid biomarker for broad application will in fact require assessment of many markers to create a panel that can support diagnosis. The lipidomic and proteomic analyses I employed in this work are approaches with the required breadth and lack of bias to be successful in such an undertaking, and I hope that the work described in this thesis provides a foundation for future development of such biomarker panels.

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Abbreviations

5HT	Serotonin
AChE	Acetylcholinesterase
ACHEi	Acetylcholinesterase inhibitor
ACN	Acetonitrile
ACRM	American College of Rehabilitation Medicine
ACTH	Adrenocorticotrophic Hormone
AD	Alzheimer's disease
ANKK1	Ankyrin repeat and kinase domain containing 1
ApoE	Apolipoprotein E
ATP	Adenosine triphosphate
AUC	Area under the curve
AUDIT	Alcohol Use Dependency Identification Test
Aβ	Amyloid β
B-H	Benjamini–Hochberg
BBB	Blood Brain Barrier
BCA	Bicinchoninic acid
BChE	Butyrylcholinesterase
BDNF	Brain derived neurotrophic factor
BTBIS	Brief Traumatic Brain Injury Screen
c-Tau	Cleaved Tau
CAM	Chloroacetamide
CBF	Cerebral Blood Flow
CC	Corpus callosum
CCI	Controlled Cortical Impact
CDC	Centers for Disease Control and Prevention
CETP	Cholesteryl ester transfer protein
CHI	Closed Head Injury
CI	Confidence Intervall
cmTBI	Complicated TBI
CNS	Central Nervous System
CNS-VS	Central Nervous System-Vital Signs
COMT	Catechol-O-methyltransferase
CRH/CRF	Corticotropin-Releasing Hormone/Factor
CSF	Cerebrospinal Fluid
CT	Computed Tomography
CTE	Chronic Traumatic Encephalopathy
DDA	Data-dependent acquisition
DEET	N, N-Diethyl-metatoluamide
DHA	Docosahexaenoic acid

DIPE	Diisopropyl ether
DP	dementia pugilistica
DPA	Docosapentaenoic acid
DRD2	D2 DA receptor gene
DSM	Diagnostic and Statistical Manual
DTI	Diffusion Tensor Imaging
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
EPC	Endothelial progenitor cells
ePL	ether Phospholipid
ESS	Epworth Sleepiness Scale
ETA	Eicosatetraenoic acid
FA	Formic acid
FA	Fatty acid
FKBP5	FK506 binding protein 5
fMRI	Functional Magnetic Resonance Imaging
FPI	Fluid Percussion Injury
GABA	γ-Aminobutyric acid
GCS	Glasgow Coma Scale
GFAP	Glial Fibrillary Acidic Protein
GR	Glucocorticoid Receptors
GWI	Gulf War Illness
GWIC	Gulf War Illness Consortium
HBB	Hemoglobin subunit beta
HCD	Higher-energy C-trap dissociation
HDL	High-density lipoprotein
HILIC	Hydrophilic interaction chromatography
HPA	Hypothalamic Pituitary Adrenal
HPRP	High pH reverse phase spin column chromatography
HRP	Horseradish Peroxidase
HTTLRP	serotonin-transporter-linked polymorphic region
ICH	International Conference on Harmonization
ICP	Intracranial Pressure
IL	Interleukin
iTRAQ	Isobaric tagging for relative and absolute quantitation
KMO	Kaiser-Mayer-Olkin
LC	Liquid Chromatography
LCAT	Lecithin:cholesterol acyltransferase
LCMS	Liquid Chromatography Mass Spectrometry
LDL	Low-density lipoprotein
LOC	Loss of Concussion
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine

MAO-A	Monoamine Oxidase A
MDA	Malondialdehyde
MDD	Major Depressive Disorder
MES	2-(N-morpholino)ethanesulfonic acid
MFI-20	Multi-dimensional Fatigue Inventory
MLM	Mixed Linear Modeling
MRI	Magnetic Resonance Imaging
MS	Mass Spectrometry
mTBI	Mild Traumatic Brain Injury
mTBI	Mild TBI
MUFA	Monosaturated fatty acid
NF	Neurofilament
NFT	neurofibrillary tangles
NPY	Neuropeptide Y
NSE	Neuron Specific Enolase
NYP	Neuropeptide Y
OEF	Operation Iraqi Freedom
OIF	Operation Enduring Freedom
p-Tau	phosphorylated Tau
PAF	Platelet-activating factor
PB	Pyridostigmine bromide
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PCA	Principal Component Analysis
PCL-M	PTSD Checklist-Military Version
PCS	Post Concussion Syndrome
PDHA	Post-Deployment Health Assessment
PE	Phosphatidylethanolamine
PER	Permethrin
PET	Positron Emission Tomography
PI	Phosphatidylinositol
PL	Phospholipid
POMS	Profile of Mood States
PSQI	Pittsburgh Sleep Quality Index
PTSD	Posttraumatic Stress Disorder
PUFA	Polysaturated fatty acid
PVN	paraventricular nucleus
r-mTBI	repeated mild TBI
RAC	Research Advisory Committee
RB	Resuspension Buffer
ROC	Receiver Operating Curve
ROS	Reactive Oxygen Species
S100β	S100 Calcium-Binding Protein β

SBDP	Spectrin breakdown product
SCID	In-source collision induced dissociation
SDC	Sodium deoxycholate
SDS-PAGE	Sodium odecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard Error of the Mean
SF-36V	MOS Short Form 36-veteran version
SFA	Saturated fatty acid
SM	Sphingomyelin
SNTF	α-spectrin N-terminal fragment
SOP	Standard Operation Procedure
SPECT	Single Photon Emission Tomography
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TBI	Traumatic Brain Injury
TCEP	Tris(2-carboxyethyl)phosphine
TEAB	Triethylammonium Bicarbonate
TGF	Transforming growth factor
TMB	3,3′,5,5′-Tetramethylbenzidine
TMT	Tandem Mass Tag
TNF	Tumor Necrosis Factor
UCH-L1	Ubiquitin C-terminal hydrolase
USAAARL	United States Army Aeromedical Research Laboratory
VA	Veterans Affairs
VAS	Visual Analog Scale
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein
WHO	World Health Organization
ZAS	Zung Anxiety Scale
ZDS	Zung Depression Scale

Chapter 1 Introduction

1.1 Epidemiology of TBI and PTSD and their comorbidity

Traumatic brain injury (TBI) is a major cause of disability and death among young adults in the US and worldwide, and its prevalence is increasing [1,2]. In 2008 the Center for Disease Control and Prevention estimated over 1.7 million occurrences of TBI in the US per year [3] and 214,000 in the UK [4] with 5.3 million Americans (1 million in the UK) living with long-term severe disabilities as a result of brain injury. Moreover, in the US up to 275,000 hospitalizations and 1,365,000 emergency department visits are due to TBI [3,6].

Besides its importance for the civilian population, TBI has been termed the "signature injury" of the wars in Iraq and Afghanistan with over 19% of returning service members diagnosed with a deployment-related TBI [7].

Economic costs of TBI account for 10% of the annual health care budget amounting to approximately US$ 76.3 billion [8,9]. Furthermore, each veteran affected by a TBI creates an average annual cost of $11,700 in medical treatment [10]. Additionally, the World Health Organization (WHO) estimates that, by 2020, TBI will become the third leading cause of disability and mortality worldwide, further raising costs in the future [11].

Faul *et al,* defined TBI as the “silent epidemic”, due to the fact that complications from mild injury may not be readily apparent [3]. Furthermore, awareness about TBI among the general population is still limited, although the recent case reports of a distinct neurodegenerative disease in American football players, termed “chronic traumatic encephalopathy” (CTE), has increased awareness of the consequences of exposure to a

history of repetitive concussions [12–15]. Other popular sports, including boxing, ice hockey, soccer, rugby, the martial arts, cycling, motor racing, equestrian sports, rodeo, skiing and roller skating also contribute to increasing numbers of TBI [16]. These together underscore the vital need to understand the underlying neurobiological mechanisms in TBI and to identify new novel biomarkers and therapeutic targets for objective diagnosis and treatment.

Severity of injury in TBI correlates with cognitive and motor function limitations. A study by Colantonio and colleagues, which investigated long-term outcomes after moderate to severe TBI, revealed that among the 306 patients evaluated up to 24 years post-injury, only 29% had returned to full-time employment and still experienced limitations in daily life [17]. Most TBIs are categorized as mild (~80%), which is the most challenging to diagnose, monitor and treat [18]. In the majority of mTBI patients neurocognitive impairment is temporary, with resolution within days to weeks post-injury and no visible evidence of degenerative changes from brain imaging tests [19]. Although studies have shown that most patients make a full recovery within 3 months of injury, additional research indicates long-term molecular and cellular damage that may persist for years with approximately 15%–25% of mTBI cases experiencing ongoing symptoms, which could cause significant disability [19,20]. These somatic, cognitive, emotional, motor, or sensory symptoms ascribed to a concussion (or following TBI) are termed post concussion syndrome (PCS).

In 2004 The World Health Organization published a detailed review of the literature consisting of 120 studies on the prognosis after mTBI. Symptoms usually fall into three categories: somatic (e.g., headache, dizziness, weakness, sensitivity to light and

sound), cognitive (e.g., difficulties with attention, memory, and language), and psychological/ behavioral (e.g., irritability, depression, anxiety, personality changes)[21]. Several factors have been suggested to be predictors of prolonged symptoms after the initial TBI insult. These include demographic factors, such as gender and age, as well as psychosocial factors, such as an inability to sustain relationships and existence of previous psychiatric disorders. External stressors, pre-existing TBI and other neurological disorders are also thought to influence the recovery process [19,22]. Especially in military settings, comorbidities can further make identification of TBI symptoms more difficult.

Military TBI patients are particularly at risk for the development of more severe, long-term psychiatric disturbances, including personality change, post-traumatic stress disorder (PTSD), anxiety, mania, substance abuse, psychosis and depression [23,24]. Due to the wars in Iraq and Afghanistan, attention has been brought to the importance of TBI in its complex relationship with PTSD, owing to their common comorbid appearance in veterans [7,25,26]. PTSD is a debilitating consequence of witnessing severe or life-threatening trauma [27] and is associated with a number of symptoms that interfere with neurological functioning and quality of life, often resulting in social withdrawal, anger and aggression, and sleep disturbance [28].

The RAND 2008 Report recorded that 14% of troops, returning from Operation Iraqi Freedom (OIF, 2003-2011) and Operation Enduring Freedom (OEF, 2001-2014), met criteria for PTSD or major depression, 19% sustained a TBI and 5% had been diagnosed with co-morbid TBI and PTSD or depression [7,25].

A national sample of all OEF/OIF Veterans who received care for TBI through the Veterans Affairs (VA) system during the year 2009 revealed in this TBI group, 89%

had a concomitant mental health disorder, with the most frequent diagnosis being PTSD (73%) [29]. Especially in the military population, PTSD and TBI often coexist because brain injuries are often sustained during traumatic events and experiences.

It has been estimated that, within the military, proper care of each veteran affected by TBI creates annual costs of $11,700, whereas a PTSD veteran creates an average annual cost of $8,300, in medical treatment [10]. Of note, patient care for comorbid TBI and PTSD costs are estimated at $13,800 in the first year.

Furthermore, PTSD is often complicated by other mental health problems [30,31]. Epidemiological studies have documented that subjects with a given diagnosis of PTSD show elevated risk for a broad spectrum of disorders, including depression, anxiety disorder, panic disorder, conduct disorder, personality disorders, and multiple types of substance abuse [32]. Returning service members often present common clinical symptoms which can be attributed to either PTSD or TBI or both [33]. A cluster of overlapping symptoms can be found in Figure 1-1, which include depression/ anxiety, insomnia, irritability/anger, trouble concentrating, fatigue, hyperarousal and avoidance. The presence of overlapping (and heterogeneous) clinical symptoms in both TBI and PTSD can make it extremely challenging for physicians to precisely diagnose both conditions either individually or if they occur together in comorbid cases. This emphasizes the need to identify an objective biomarker that can distinguish between those two conditions.

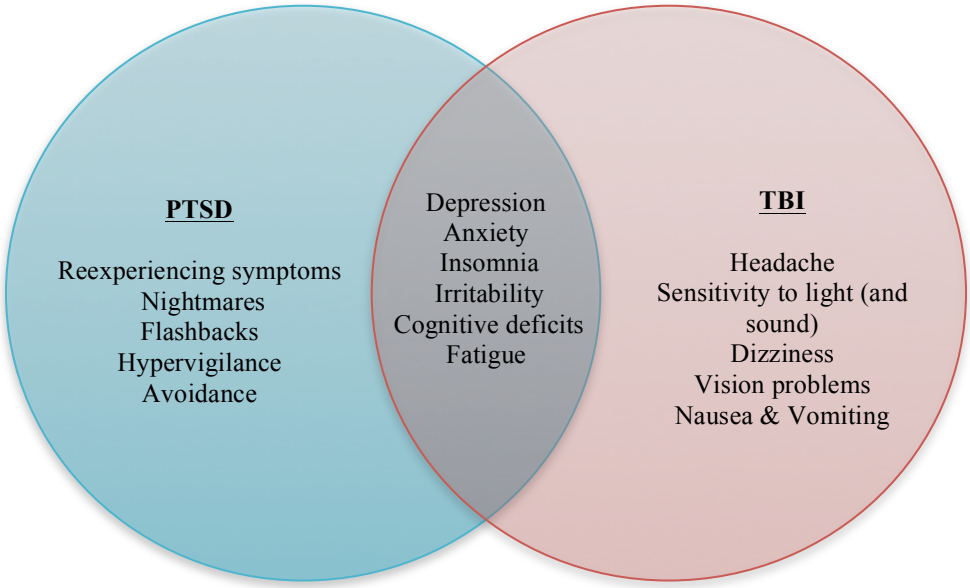


Figure 1-1: Overlapping persistent symptoms of PTSD and TBI (adapted from Stein & McAllister, 2009). [33]

1.2. Clinical diagnosis

1.2.a TBI

Due to the heterogeneous presentation of TBI, specific diagnoses for different severities is of importance in order to determine suitable treatments for the patient's needs, including rehabilitation programs [34]. As such, accurate identification of those TBI patients is a major challenge in clinical settings. The Department of Defense and Veteran's Affairs define a TBI as a traumatically induced structural injury and/or physiological disruption of brain function. This injury is the result of an external force that leads to a new onset/worsening of clinical signs, which can be described as the following[35]:

1) LOC: Any period of loss of or a decreased level of consciousness
2) PTA: Any loss of memory for events immediately before or after the injury (post-traumatic amnesia)

3) AOC: Any alteration of consciousness/mental state at the time of the injury (confusion, disorientation, etc.)
4) Neurological deficits (weakness, loss of balance, change in vision, praxis, paresis/plegia, sensory loss, aphasia, etc.)
5) Intracranial lesion

The presence and duration of TBI symptoms defines the severity level, which range from mild to moderate to severe TBI. The different severity levels of TBI are associated with different durations of loss of consciousness (LOC), length of posttraumatic amnesia and alteration of consciousness, as well as normal/abnormal structural imaging. The joint Veterans Health Administration/Department of Defense clinical practice guidelines classify TBI severity on the basis of the American College of Rehabilitation Medicine (ACRM) definitions [36]. Table 1-1 presents the guidelines for TBI severity classification. The pathophysiology for mild TBI is different than for moderate and severe TBI, mostly distinguishable through the absence of structural/anatomical damage, whilst similar bimolecular process are activated in the brain in all cases of TBI (see below, pathophysiology of TBI). Overall, TBI classification is based of cause and symptomology, as well as existence of comorbid disorders (see above), rather than underlying biological mechanisms.

	Glasgow Coma Scale [37]	Loss of Consciousness	Posttraumatic Amnesia	Alteration of Consciousness	Structural Imaging
Mild TBI	13-15	0-30 minutes	0-1 day	<24 hours	Normal
Moderate TBI	9-12	30min - 24 hours	1-7 days	>24 hours	Normal/ Abnormal
Severe TBI	<9	>24 hours	>7 days	>24 hours	Normal/ Abnormal

Table 1-1 Severity level of TBI defined on the bases of the ACRM definition. Mild TBI is defined by LOC of 0-30min, posttraumatic amnesia for up to 1 day, alteration of consciousness under 24hours, normal structural imaging and a score of 13-15 in the Glasgow Coma Scale (GCS; described below). Moderate TBI is defined via LOC of 30min-24hours, alteration of consciousness over 24hours, 1-7 days posttraumatic amnesia and a GCS score of 9-12. Severe TBI shows LOC over 24hours, alteration of consciousness over 24hours and posttraumatic amnesia over 7 days with GCS below 9. Both moderate and severe injury can show normal or abnormal structural imaging.

Glasgow Coma Scale

Among clinicians, the GCS is the most common test used for TBI. It is a 15-point test, 3 being the worst score, which evaluates injury severity and level of consciousness after TBI. The GCS tests eye, verbal and motor responses, immediately after hospital admission and during the acute phase of examination and care. The three broad categories as shown in Table 1-1 classify TBI into: severe TBI (GCS 3-8), moderate TBI (GCS 9-13) or mild (GCS 14-15) [38] (Table 1-2 (adapted from http://glasgowcomascale.org)).

Criterion	Response	Score
Eye Opening Response	Spontaneous	4
	To sound	3
	To pressure	2
	None	1
Verbal Response	Oriented/normal conversation	5
	Confused/disoriented conversation	4
	Words	3
	Sounds	2
	None	1
Motor Response	Obeys commands	6
	Localizing (to pain)	5
	Normal flexion (withdraw to pain)	4
	Abnormal flexion	3
	Extension	2
	None	1

Table 1-2: Glasgow Coma Scale scoring

Although the GCS is widely used, it has several drawbacks relating to the scoring process itself. For example, eye response is difficult to test if eyes are swollen shut from the injury, a person's verbal responses cannot be scored if the person drank alcohol before his injury as alcohol may make his speech hard to understand or motor response might decline if an injury causes pain with movement, or makes the person unable to move. Additionally, the GCS does not check if a person can learn and remember new things, which is an important factor helping caregivers predict recovery after a TBI. Moreover, medical sedation, paralysis, intoxication through drugs, as well as other injuries that distract from TBI, can confound the resulting score of the GCS [38,39]. Multiple epidemiological studies have shown that those confounders are increasing, which leads to a decrease in the value of the GCS [40]. It also has been shown that the GCS is most precise in the severe TBI population, however has poor performance for mTBI, which is 80-90% of all TBI cases [39]. A low GCS score is associated with acute mortality and morbidity, but

higher scores lack the prediction of long term outcome, providing no information about underlying neurological deficits [41].

Overall the GCS was introduced for clinical monitoring following TBI, but was then adapted to diagnose TBI severity. Although it is widely recognized that the GCS lacks adequacies for this purpose, no real alternative exists. There is no consensus for the selection of clinical features or standardized test for TBI diagnosis and severity grading and TBI subtype definition, which often is bases on findings from brain imaging studies, history and clinical features[42].

Neuroimaging techniques have been used for the assessment of more severe TBI to add to the value of GCS and gather information about structural and functional pathological mechanisms. Examples include detection of skull fractures and hematomas through computed tomography (CT), visualization of macroscopic areas of white matter damage via magnetic resonance imaging (MRI) and identification of functional metabolic changes via functional MRI, positron emission tomography (PET), single-photon emission computed tomography (SPECT) and diffusion tensor imaging (DTI), just to name a few. Diffusion tensor imaging in particular has shown promising and consistent findings for sports-related mild TBI in older adolescents and young adults in the post-acute period and decreases in the integrity of white matter microstructure have shown to occur 3 or more months after mild to severe pediatric TBI [43]. Overall, neuroimaging techniques allow partial diagnosis of TBI severity and play an important role in determining the presence and extent of the injury and guide surgical planning that require a minimum of invasive interventions.

However, the techniques also come with general drawbacks, such as high cost, poor availability and the lack of ability to perform a secondary scan in short term follow up. Especially in military settings, such equipment is often not available on site. Using brain-imaging techniques for diagnosis of TBI is especially challenging in cases of mTBI.
It is of importance to note that these Department of Veterans Affairs (VA) and the Department of Defense (DoD) guidelines were designed to provide information and assist in decision-making. They are not intended to define a standard of care. The classification of the severity of a TBI helps guide acute management and establishes selection criteria for admission to intensive care units. Furthermore, whilst it has been shown that severe TBI (and mostly moderate TBI) is related to long-term cognitive defects there is insufficient evidence to determine a relationship between as single, mild classified TBI and long-term consequences, although studies of post concussive syndrome have increased. It has been shown that TBI severity classification such as LOC, PTA, AOC, the GCS-score and abnormalities on CT scanning in the acute stage predict short term outcome rather than long term outcome[44].

Various clinical trials have been conducted on neuroprotective agents for TBI, usually in randomized controlled trials. Yet, up till now, all multi-center trials have failed to successfully produce results. The commonly used categorization of TBI via the "artificial" GCS has been named as an error introducing factor, separating injury into categories, instead of using the fact that clinical severity is based on a pathophysiological continuum. Underlying biochemical mechanisms occur across the spectrum of clinical severity, but might differ in frequencies or strength of biological signals. Furthermore, as mentioned above, patients with the same GCS can have different types of injury.

Therefore a new gold standard needs to be implemented for the diagnosis of TBI and its pharmaceutical outcome measures (Glasgow outcome scale) that allows for a more precise categorization of TBI in clinical trials. Objective biomarkers or mechanistic targeting is based on the identification of occurrence/time of pathophysiological mechanisms. Additionally, individualized personal treatment in a clinical trial, instead not depended on the used clinical diagnosis[45]. Overall, diagnostic measures need to be improved in order to validate clinical trial outcome.

1.2.b PTSD

Post-traumatic stress disorder criteria and symptoms have been defined in the latest Diagnostic and Statistical Manual (DSM)-Fifth Edition-Text Revised (DSM-5)[46] and reviewed by Kennedy et al. [47]. It is a trauma and stressor-related disorder, which requires exposure or witness to a traumatic or stressful event as a diagnostic criterion, which is threatening to safety, and one must respond to this event with fear, horror, or helplessness. The DSM-5 diagnosis of PTSD requires four major symptom criteria: (1) Intrusion (re-experiencing of the trauma, e.g. as nightmares or intrusive memories), (2) Avoidance, (3) negative alterations in Cognition and Mood (persistent and distorted blame of self or others, and persistent negative emotional state), and (4) alterations in Arousal and Reactivity (reckless or destructive behavior). In the case of avoidance, patients must present at least three avoidance symptoms (active avoidance of thoughts, feelings, or reminders of the trauma, inability to recall some aspect of the trauma, withdrawal from others, or emotional numbing). Lastly, arousal must be present in form

of insomnia, irritability, difficulty concentrating, hypervigilance or heightened startle response [47].

Like the GCS for the diagnosis of TBI, the DSM 5 diagnostic criteria for PTSD rely on observation rather than an objective measure, leading to limitations that mirror those of the GCS. Besides the DSM5 criteria listed above, which focus on behavioral presentation, functional neuroimaging techniques have been used to investigate PTSD, such as functional MRI (fMRI), SPECT and PET. Measures such as blood oxygenation level for fMRI, or regional cerebral blood flow/regional cerebral metabolic rate for glucose for PET, evaluate regional activity in the brain [48]. Although sensitivities in spatial resolution have increased for imaging techniques, the high comorbidity between PTSD and other anxiety disorders (e.g. depression) makes diagnosis difficult and leads to a lack of sensitivity. As such a more objective method/biomarker is needed for more precise diagnosis [48].

For military populations the Post-Deployment Health Assessment (PDHA), the Brief Traumatic Brain Injury Screen (BTBIS), Neurobehavioral Symptom Inventory (NSI), as well as the PTSD Checklist-Military Version (PCL-M) are, among others, the standard for TBI and PTSD assessment. A copy of the questionnaires can be found in the Appendix.

A downside commonly exhibit to psychiatric diagnostic categories is the considerable symptom heterogeneity. PTSD shows symptom overlap with generalized anxiety disorder (GAD) and major depressive disorder (MDD), as well as a wide range of fear and anxiety disorders, such as panic disorder, social anxiety disorder, and specific phobias. In order to reduce heterogeneity in PTSD, mechanisms responsible for subtypes of symptoms need

to be investigated, as well as longitudinal data needs to be acquired, collected after the traumatic event to enable early prediction and classification of these conditions allowing to identify targets for prevention.

1.3 Pathophysiology of TBI

Traumatic brain injury has been defined as a brain injury caused by an external mechanical force, e.g. a bullet or blow [49], significantly disrupting brain function as indicated by any of the following: a period of loss of consciousness or alteration in consciousness (e.g., confusion, disorientation); loss of memory (amnesia) for events immediately before or after the injury; neurological deficits (e.g., weakness, loss of balance, change in vision); or intracranial lesion [50].

Damage caused by TBI is the result of a contact and/or acceleration/deceleration injury type mechanism [51]. Contact injury, which is usually associated with focal brain injury, is a result of an object hitting the head, the consequences of which can include skull fractures, intracranial hematomas, lacerations, contusions, penetrating wounds and increased intracranial pressure (ICP) [52,53]. Acceleration/deceleration brain injury, which leads to tensile, shear and compressive strain, results from unrestricted head movement. These injury mechanisms are associated with diffuse brain damage, such as ischemic brain injury, diffuse brain swelling and axonal damage [51,54].

Pathology of the injury is dynamic and can be divided into 2 subcategories. It begins with the mentioned (1) primary injury (mechanical), which occurs at the moment of trauma. Following primary insult (2) a secondary phase of TBI is initiated; a cascade of biochemical changes, that is attributable to further cellular damage from the effects of

primary injuries and develops over hours and days. This includes elevated neurotransmitter release and subsequent activation of receptors and subsequently to early ionic changes in postsynaptic membranes, calcium-mediated damage, mitochondrial dysfunction, free-radical generation and inflammatory responses as well as mechanical damage [55]. The result of the described metabolic crisis and excitotoxicity includes membrane degradation and blood-brain barrier (BBB) compromise and changes in vascular permeability, and eventually leads to an unregulated transit of immune molecules in and out of the injured brain [56,57]. Figure 1-2 shows an overview of pathways associated with the secondary injury response after TBI [58].

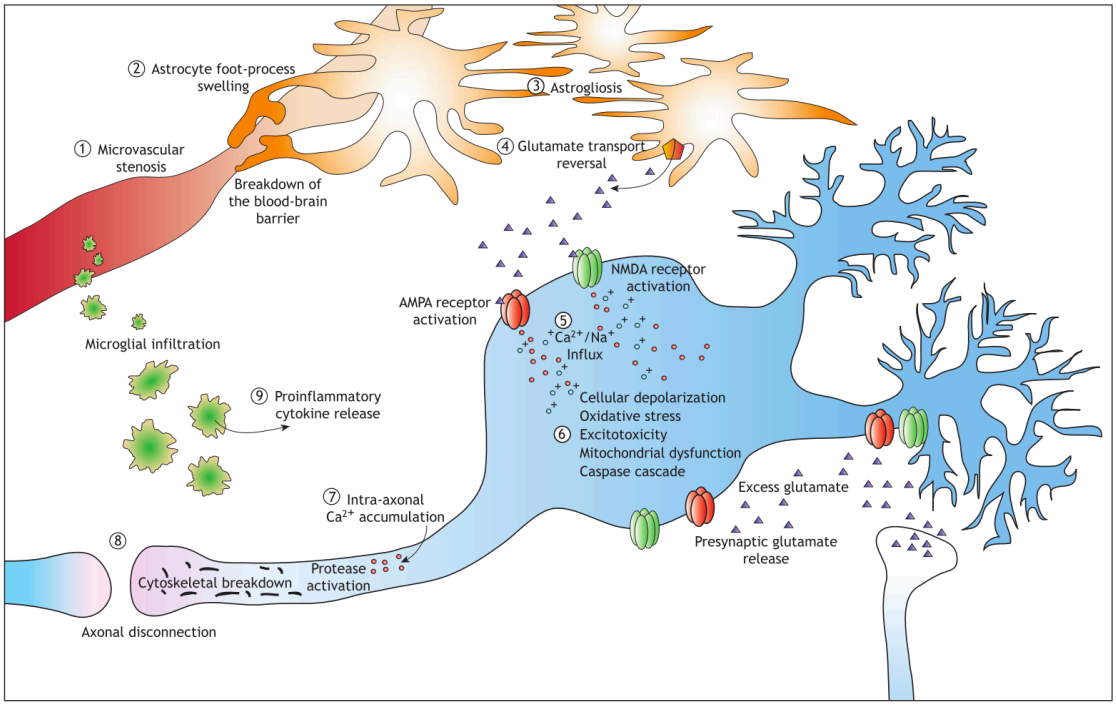


Figure 1-2 Pathways associated with secondary response to TBI (image from [58]. (1) TBI leads to microvascular stenosis, (2) as well as swelling of astrocyte foot processes, which may lead to blood–brain barrier break down. (3) Astrogliosis results in (4) a reversal of glutamate uptake and depolarization at the neurons through excitotoxic mechanisms. (5) Calcium influx, due to white and grey matter injuries, leads to molecular cascades. (6) This results in generation of free radicals, excitotoxicity, mitochondrial dysfunction, caspase activity and modifications at postsynaptic receptors. (7) When calcium influxes and accumulates in the axons, proteases are activated, which leads to a degradation of the cytoskeleton (8). Secondary injury is also accompanied by a release of proinflammatory cytokines. (9) These cytokines activate cell-death cascades or postsynaptic receptor modifications.

Genetic influences on outcome after TBI

Besides physiological variations in humans such as age, sex and fitness, genetic predisposition has been suggested to influence TBI recovery, because patients matched on basic demographics can exhibit vastly different outcomes as a result of TBI than others sustaining a comparable injury.

Multiple gene candidates have been proposed and extensively reviewed [59], for example genes that influence the extent of injury, such as the release of pro-and anti-

inflammatory cytokines [60]. These include variations within the tumor necrosis factor α (TNFα) gene, as TNFα is involved in mediating cell death, as well as promotion of neuronal repair at later stages [61] and genetic variations in IL-1 and IL-6 family and their receptors [62].

Furthermore, genes that influence the dopaminergic system, such as variations in the Dopamine Receptor D2 (DRD2) and Ankyrin repeat and kinase domain containing 1 (ANKK1) (controls synthesis of dopamine in the brain) genes have been proposed to influence TBI [63]. Additionally, polymorphisms in genes which are part of the serotonergic system (including serotonin-transporter-linked polymorphic region (5-HTTLPR), which encodes a transporter protein that removes serotonin (5HT) from the synaptic space and monoamine oxidase A (MAO-A), which encodes an enzyme involved in breakdown of serotonin, dopamine and norepinepherine), are potential candidates [59,64]. Finally, variants in the brain derived neurotrophic factor (BDNF) gene, which encodes proteins involved in survival, growth and differentiation of neurons, have been suggested to influence TBI outcome [65].

In addition to those listed above, many gene variants have been proposed to influence TBI recovery, none however has been as intensively studied as the Apolipoprotein E (gene: APOE; protein: apoE). Under stress, ApoE is produced by astrocytes, microglia and neurons in the central nervous system (CNS) [66]. Apolipoproteins are lipid carrier proteins that transport and redistribute lipids among cells and play a role in growth and repair of neurons, neuronal protection, mediation of brain inflammatory processes and maintenance of synaptodendritic connections [67]). The gene encodes three common alleles (ε2, ε3 and ε4), leading to six common genotypes

including three heterozygote ε3/ε2, ε3/ε4, ε4/ε2 and three homozygote genotypes ε2/ε2, ε3/ε3 and ε4/ε4 [68].

APOE has long been associated with Alzheimer's disease (AD), more precisely APOE ε4 was identified as a risk factor for AD [69], before a link to TBI outcome was made. This is of importance due to the possible link between APOE, AD and TBI [70]. Prior to the identification of APOE ε4 as a risk factor for AD, autopsy studies had shown that boxers who had sustained repeated head trauma showed at autopsy similar brain pathology to those of AD subjects [71]. Additionally, 30% of postmortem brains from patients sustaining a single severe TBI and surviving less than two weeks showed amyloid beta (Aβ) deposition, a classical hallmark of AD neuropathology [72]. Since it was observed that APOE genotype influences Aβ accumulation in AD brain (with e4 carriers showing higher levels of Aβ) [73] it is logical to assume that APOE and TBI outcome are connected.

In order to determine if APOE ε4 plays a role in severity of TBI outcome, meta-analyses have aimed to evaluate the connection. Zhou and colleagues performed a meta-analysis in 2008, including 100 studies conducted in the years between 1993 and 2007 [74] encompassing TBI severities ranging from mild to severe. The group concluded that APOE e4 is not involved in initial severity of brain injury, but with an increased risk of poor TBI outcome after 6 months post injury. A study in 2015 included 12 studies published since 2003, confirmed and extended the association of APOE ε4 and an increased risk for functional TBI outcome for over 6 month [75]. Ponsford and colleagues detected this association even for 1 and 5 years post injury [76].

Although the exact underlying biological mechanisms are not completely understood, it appears that the ε3 isoform is neuroprotective, whilst ε4 was shown to have a higher binding avidity to Aβ peptides promoting aggregation [77]. Furthermore ApoE4 has been associated with a less efficient transport of lipids [78,79], an increase in inflammation [80–82], poorer protection against oxidative injury [83], mitochondrial dysfunction [84], and diminished growth and branching of neurites resulting in poorer repair [85,86].

1.4 Pathophysiology of PTSD

The above-described symptoms of PTSD are present for an extending period of time and therefore seem to display persistent, abnormal functions of neurobiological systems to the stress of exposed and witnessed trauma [87]. Systems that regulate stress responses, and are thus candidates for PTSD pathobiology, include neuroendocrine and chemical neurotransmitter pathways, as well as neuroanatomic brain regions that regulate fear.

1.4.1.a Neuroendocrine responses

Immediate neuroendocrine responses to stress involve increases in heart rate and blood pressure ("fight or flight" reaction). This response allows a greater perfusion of blood glucose to muscles and vital organs and results in increased energy to skeletal muscles, allowing the organism to better fight or flee adverse situations. At the center of endocrine factors influencing PTSD are dysregulation of cortisol and thyroid hormones, regulated via the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is one of the

principal effectors of the stress response in the human body, responsible for the maintenance of homeostasis after challenge, but also for supporting baseline functioning. It is localized in the paraventricular nucleus (PVN) of the hypothalamus, the anterior lobe of the pituitary gland, and the adrenal gland. This collection of structures is commonly named the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1-3).

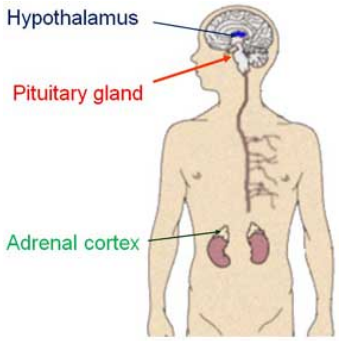


Figure 1-3 Location of the hypothalamic-pituitary-adrenal (HPA) axis in the human body (Image from http://www.simplypsychology.org).

Figure 1-4 shows the basic function of the HPA axis [88]. The PVN synthesizes and secretes corticotropin-releasing factor (CRF), also known as corticotrophin-releasing hormone (CRH), the principal regulator of the HPA axis; increased levels of CRH due to stress results in HPA axis activation [89,90]. After secretion, CRH is transported to the anterior pituitary, where it binds to CRH1 receptors, causing the release of adrenocorticotrophic hormone (ACTH) into the blood stream. There, ACTH stimulates the adrenal cortex to synthesize and release the glucocorticoids cortisol (humans) or corticosterone (rodents). Amongst other functions, cortisol serves to increase glucose availability to be utilized as an energy resource during times of stress [91]. Feedback inhibition of the HPA axis is mediated in part by the binding of cortisol to glucocorticoid receptors (GRs) at the level of the hippocampus, hypothalamus and pituitary to dampen

stress-induced activation of the HPA axis [88]. Modulation of the CRH response to threat relies on a complex feedback system involving the glucocorticoid receptors (GCCR, GCR2) and regulating genes (e.g., FK506- binding protein 5 (FKBP5)). Because PTSD is characterized by dysregulation of the stress response system, alterations in the HPA axis functionality, especially a hyper-responsive cortisol feedback regulation, have been implemented in this disorder [92,93]. This dysregulation is in part due to enhanced sensitivity of the glucorticoid receptor (GR)-mediated feedback mechanism that suppresses stress-induced cortisol release. The receptor mediates many of the effects of GCs on target tissues via direct binding to hormone-responsive elements on the DNA or via interactions with other transcription factors resulting in a modulation of gene transcription [94].

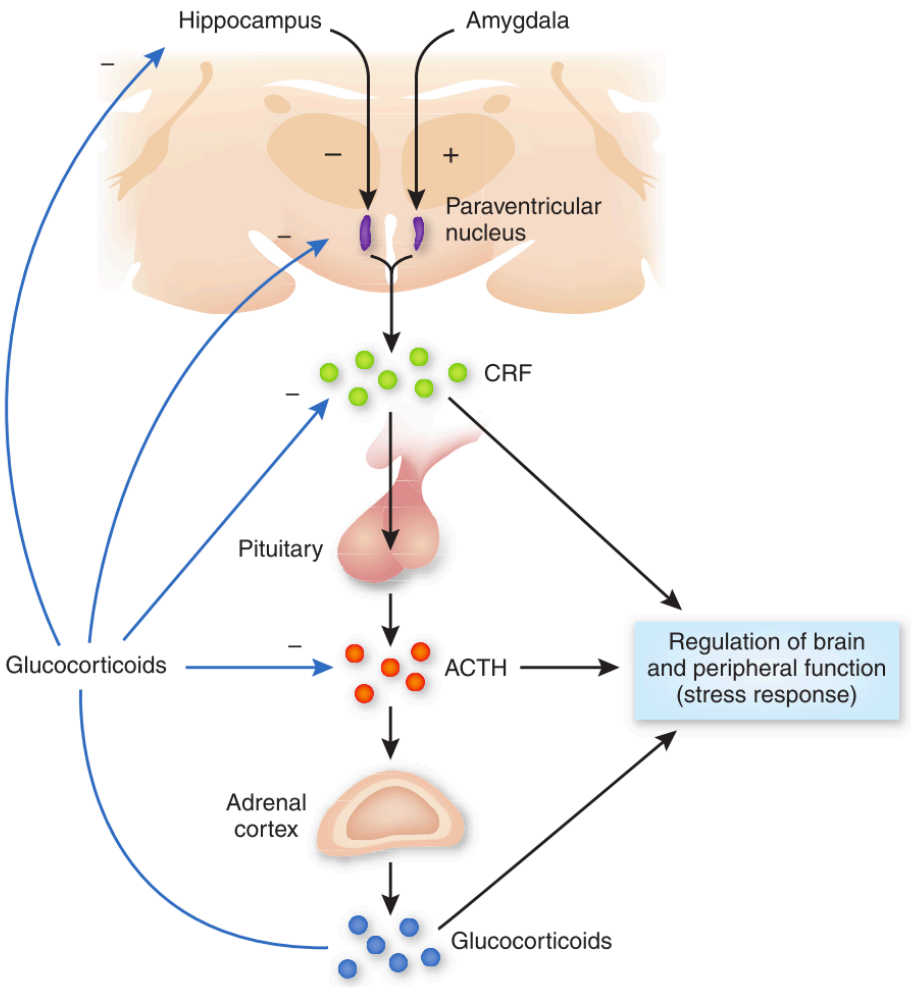


Figure 1-4: HPA axis function. The paraventricular nucleus in the hypothalamus, releases CRH (CRF) that is transported to the anterior pituitary. There, it causes the release of ACTH into the blood stream. ACTH stimulates the adrenal cortex to synthesize and release the glucocorticoids cortisol (humans) or corticosterone (rodents). Glucocorticoids feed back at the level of the hippocampus, hypothalamus and pituitary to dampen excess activation of the HPA axis (Image source: Steven E Hyman and Kim Ceasar 2009 [88]

1.4.1.b Neurochemical factors

Besides endocrine factors, neurochemical factors are part of the pathophysiology of PTSD, as they are found in brain circuits, which influence stress and fear responses. This includes catecholomine, amino acids, serotonin, peptides and opioid neurotransmitters (Note: catecholamine and serotonin (as well as acetylcholine)

dysregulation is also found in patients with TBI, as a result of diffuse axonal injury [87]). PTSD subjects have been reported to demonstrate abnormal regulation of catecholamines (increased dopamine and norepinephrine levels), serotonin (decreased concentrations), amino acids (decreased ϒ-aminobutyric acid (GABA) activity, increased glutamate), peptide (decreased plasma NPY concentrations and increased cerebrospinal fluid (CSF) β -endorphin levels [87]).

1.4.1.c Brain circuitry – Neuroanatomic changes

Changes in brain structures were observed in PTSD patients that mediate adaption to stress and fear conditioning [95–97]. These neuroanatomical changes include reduced volume and activity in the hippocampus, increased activity in the amygdala and reduced prefrontal and anterior cingulate volume, as well as decreased medial prefrontal activation in the cortex [87,96,97].

1.4.2 Genetic influences on PTSD

One of the key questions in trauma research is why certain individuals develop PTSD after a traumatic event, whilst others who experienced the same or similar trauma are resilient. Genetic influences on PTSD risk have received much attention, although only limited progress has been made in identifying the genetic variants that could explain the different susceptibility to trauma among humans.

There have been numerous genetic association studies examining the role of various genes in PTSD that play a role in the described neurochemical and neuroendocrine responses (extensively reviewed in Cornelis & Nugent 2010). This for

example includes genes relevant to the HPA axis, such as the mentioned FKBP5, which mediates GR translocation [98,99]. Other potential gene candidates are serotonin transporter genes [100,101], as PTSD can be treated by targeting the serotonin transporter (SLC6A4) with selective-serotonin reuptake inhibitors (SSRIs), and genes involved in the regulation of the dopaminergic system (e.g. D2 DA receptor gene (DRD2) or catechol-O-methyltransferase (COMT)). Finally, genes related to neurotransmission and neuromodulation have been under investigation in PTSD. An example is the brain BDNF gene, also of interest and relevance in TBI as discussed above. BDNF plays an important role in hippocampal-dependent learning and is involved in the neural plasticity underlying the extinction of fear and recovery from stress (Chhatwal et al., 2006; Heldt et al., 2007; Soliman et al., 2010). Many other genes have been suggested to contribute to the development of PTSD, such as neuropeptide Y (NPY, thought to have a role in decreasing fear states and enhancing fear extinction [102]), monoamine oxidase B (MAO-B, which catabolizes dopamine, tyramine, tryptamine and other monoamines [103]), as well as the inhibitory neurotransmitter GABA [104]). Overall, further work is required to validate genetic influences on PTSD and understand their interaction [105]).

It is of importance to note that case-control candidate gene association studies in psychiatric disorders have resulted in many findings but produced very few consistent replications. This is due to the heterogeneity of the conditions, the often-small sample sizes used, multiple test performances and the low prior odds of association. Furthermore population stratification becomes an issue, due to the fact that observed significance could be an artifact of ethnic variations in allele frequencies.[106].

In recent years the concept of quantitative endophenotypes has gained attention. It uses developing traits that directly index the underlying pathology and can be measured in affected and unaffected individuals, therefore facilitating investigations of genetic influences in TBI and PTSD[107]. The study of endophenotypes involves quantitative trait analysis in large randomly ascertained families, which facilitates the investigation of genetic influences in conditions as these two strategies suggests that the quantitative trait is correlated with disease by way of the underlying liability.

Although it has been agreed upon that that certain pathophysiological systems are effected in most PTSD subjects, the myriad of trauma possibilities that causes PTSD, as well as the large amount of possible underlying mechanisms in conjunction with various genetic backgrounds, leads to the fact that not all PTSD subjects share the same exact pathophysiology, making treatment development difficult.

Additionally, in military settings TBI is often the result of a traumatic event, therefore, the epidemiologic relationship between TBI and PTSD is not a surprise. It has been suggested that damage to the brain compromises neuronal circuitry for fear regulation [108,109]. For example, functional PTSD studies revealed decreased activation in the hippocampus, and structural studies reported reduced volume in the frontal and prefrontal cortex as well as hippocampus and amygdala. It has been shown that the hippocampus is especially vulnerable to mTBI resulting in axonal damage. Furthermore, imaging studies have observed abnormalities in prefrontal and temporal brain regions in both disorders (reviewed by [109]).

The neurochemical abnormalities found in PTSD, such as changes in the previously mentioned catecholamine and serotonin, have also been observed in patients diagnosed

with TBI, presumably as a result of diffuse axonal injury [87]. The fact that common systems are affected in these conditions contributes to the co-morbidity of TBI and PTSD, which in turn makes it difficult to make an objective diagnosis. Furthermore, other mental disorders such as anxiety and depression, which show comorbidities with PTSD and TBI as well, add to the problem of finding a diagnostic measure.

1.5 Suggested Biomarkers and their Clinical Relevance and Limitations in TBI and PTSD

A biomarker is defined as a "characteristic trait that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [110]. In attempting to identify biomarkers for these complex conditions, it is first important to adequately define the goal. Biomarkers are used for multiple purposes, such as:

Diagnostic biomarkers: Identification of patients with a disease or abnormal condition

Prognostic biomarkers: Survival prediction

Pharmacodynamics biomarkers: Assessment of drug safety and evaluation of target engagement, as well as immediate consequence on biological processes

Predictive biomarkers: Identification of patients who are likely to benefit from a treatment.

Surrogate (therapeutic) biomarkers: Prediction of outcome to a therapy response

Theragnostic biomarkers: Can be used to identify and monitor the specific biochemical effect, or mode of action, of a drug, and/or to identify and monitor effects on pathogenic

processes downstream of the drug target. Therefore, *theragnostic biomarkers can* indicate whether a TBI patient is likely to benefit from a treatment in clinical trials.

In this thesis the first priority is the identification of diagnostic biomarkers, however, such biomarkers could have additional value in some of the other categories. The heterogeneity of TBI in terms of severity, symptom presentation and post-mTBI complications emphasizes the need for accurate and objective biomarkers that can classify, diagnose and monitor disease state and its progression. Furthermore, there is a need for temporal biomarkers in order to observe injury progression over time, which could lead to the discovery of different biomarkers at various stages post-insult. This is also valid for PTSD, where self-reported symptoms and co-morbidities with other psychological disorders make an accurate diagnosis difficult. Identification, validation and application of biomarkers could help ensure that patients receive the best possible therapeutic strategies, thereby avoiding unnecessary treatments, eventually reducing total health costs.

Biomarkers can come from various sources, such as brain tissues, CSF and blood. Advantages and disadvantages found for each one will be described below in section 1.7. The term "biomarker" can have a wider definition, as in the case of imaging biomarkers for example. In this thesis the term "biomarker" is generally used to describe a specific molecule or molecules that can be identified and quantified in blood.

1.5a TBI biomarker research

In the case of TBI, there is no definitive and rapid diagnostic test, unlike some other organ-based diseases such as liver or kidney dysfunction. Initially, TBI biomarker research focused on the structural damage caused by TBI (imaging) and/or investigated specific molecules linked to parenchymal damage that are present in cerebrospinal fluid or blood (albeit represented at lower levels). Imaging biomarkers such as raised ICP or inadequate cerebral perfusion pressure have proven useful for TBI diagnosis when structural changes in the brain are being observed. However, due to the focus of this thesis, this section will focus on fluid biomarkers and their use in mTBI.

After TBI, brain derived markers can be found in increased concentrations in CSF and plasma due to cell damage and disruption of the blood brain barrier (BBB) [111]. However, in mild injuries, where imaging results are negative and symptoms are non-specific, the challenge arises to objectively diagnose TBI. Many molecules have been suggested as potential biomarkers for TBI; below are reviewed the most clinically studied biomarkers along with their relevance to mTBI. Table 1-3 provides an overview of those candidate markers in acute/subacute mTBI.

Biomarker	Injury Mechanism	Location	Results for mTBI
S100 calcium-binding protein β (S100β)	Astroglial Injury as a cellular sequelae to primary insult/BBB disruption	Astrocytes, Oligodendrocytes, Extracerebral cell types	Increased in serum/CSF, results confounded by release from extracerebral tissues
Glial Fibrillary Acidic Protein (GFAP)	Astroglial Injury/BBB disruption	Astrocytes	Elevated in serum/CSF, correlates with brain imaging, promising for acute/subacute stage post injury
Neuron Specific Enolase (NSE)	Acute neuronal damage	Neuronal cytoplasm Erythrocytes, Oligodendroyctes, Platelets neuroendocrine cells	Increased in serum/CSF, low specificity/sensitivity, sensitive to lysis of red blood cells
Ubiquitin C-terminal hydrolase (UCH-L1)	Neuronal loss and disruption of the BBB	Neuron	Elevated in blood/ CSF, promising for acute/subacute phases post injury
AlphaII-spectrin breakdown protein	Axonal Injury	Neuron	Increases acutely in blood, can reflect multiorgan damage
Tau protein	Axonal Injury/ hyperphosphorylation resulting in formation of CNS tangles "tauopathy	Axon	In CSF of patients with multiple head injuries, promising marker for acute/subacute phases post injury
Neurofilaments (NF)	Axonal Injury	Axon	Findings only for NF-L in mild TBI cases (CSF), peak 4-10 days
NF-heavy (H) NF-light (L)			
Amyloid β42 (Aβ42)	Axonal Injury	Axon terminals	Elevated in plasma for up to 90 days after complicated mild TBI

Table 1-3 Candidate markers of acute mild traumatic brain injury.

Astroglia Injury

1. S100 calcium-binding protein β (S100β)

S100β is the most extensively studied biomarker for TBI. It is a low molecular-weight calcium binding protein, playing an important role in calcium regulation at intracellular levels. First thought to be specific to astrocytes, it was later shown to be expressed in oligodendrocytes, Schwann cells, as well as extracerebral cell types (e.g skeletal muscle, bone marrow cells, adipocytes and chondrocytes [112–114].

S100β is released after brain injury, through BBB opening, to the perivascular space, leading eventually to elevated levels in serum [115]. It does not cross the BBB under normal physiological conditions, with its release depending on the disruption of the BBB integrity [116]. As such, it is more appropriate as a marker for BBB-permeability [117]. The application of serum S100β protein as a clinical biomarker and outcome-predicting factor in mild TBI also meets several obstacles given that numerous factors and pathological conditions can influence baseline serum S100β concentrations. Geyer and colleagues have shown that one of the most influential factors in determining normal baseline serum levels of S100β is age [118]. Furthermore they demonstrated that S100β does not discriminate between symptomatic and asymptomatic children with minor head injury, showing the limitations in sensitivity. Although multiple studies have shown the value of S100β [119], its main limitation is its lack in sensitivity to mild and chronic brain injury [112]. Furthermore, studies have shown lack in specificity due to observed elevation of S100β in plasma of bone fracture patients [120–122].

2. Glial Fibrillary Acidic Protein (GFAP)

Glial Fibrillary Acidic Protein is a monomeric intermediate filament protein, which is part of the astroglial skeleton, believed to be only expressed by astroglia [114]. Like S100β it is a marker for astrocyte reactivity expressed across numerous subsets of astrocytes. GFAP measurements in the blood have provided promising data for injury pathway indication [123] and prediction of morbidity and mortality [124–126]. A study by Papa et al. evaluated levels of serum GFAP in mild and severe TBI and demonstrated its potential use as a biomarker up to 4 hours post injury [127]. Furthermore, Papa and colleagues recently published temporal profile data from a cohort of 1831 subjects (mild to moderate TBI and controls) investigating levels of serum GFAP (as well as ubiquitin C-terminal hydrolase (UCH-L1), see below). They observed that GFAP peaked within 24hours but was consistently raised across 7 days post-injury in mild to moderate TBI patients before returning to baseline; increased sensitivity and specifity was achieved when combined with UCH-L1 [128]. Finally in the most recent study by Bogoslovsky et al., temporal profiles (24 hours, 30 days and 90 days post injury) of mTBI patients that showed traumatic intracranial injury on CT scans and of moderate to severe TBI subjects, showed increases in plasma GFAP (as well as amyloid β (Aβ42) and tau) up to the longest (90 day) timepoint [129]. Thus, GFAP is a potential candidate for the acute and subacute phase after injury.

Neuronal Injury

1. Neuron Specific Enolase (NSE)

Neuron-specific enolase is an isozyme of a glycolytic enzyme localized primarily to the neuronal cytoplasm [114], but is also expressed in erythrocytes and oligodendroyctes [130]. It has been associated with prediction of outcome after TBI, especially in severe TBI patients. However, lower sensitivities and specificities than S100β were observed for brain injury [131,132]. The increase in levels of NSE in CSF observed in response to lysis of erythrocytes (hemolysis; contamination of CSF with blood during lumbar puncture) is a major limitation of this biomarker in peripheral blood as well as in CSF [133].

2. Ubiquitin C-terminal hydrolase (UCH-L1)

Ubiquitin C-terminal hydrolase is involved in removal and addition of ubiquitin from proteins. During normal and pathological conditions it plays an important role in removal of misfolded, oxidized and excessive proteins [134–136]. Originally used as a histological marker for neurons it was first shown to be significantly elevated in CSF and serum of severe TBI patients, with detection lasting for 1 week post-injury [137,138]. Another plasma study showed increases in UCH-L1 within one hour of injury in moderate to severe TBI patients, but not in mTBI [139]. Moreover, Papa et al. were able to distinguish concussion patients (mTBI) from non-injured and non-head injured trauma control patients [127,140]. Furthermore, a cohort study of 206 patients with TBI (r mild (83%), moderate (4%) or severe (13%) TBI) investigated UCH-L1 (and GFAP) plasma concentrations within 24 hours of injury [141]. They observed that in moderate to severe TBI patients UCH-L1 levels were higher than in mTBI subjects. Furthermore, increased

levels were also higher in mTBI with abnormal than normal CT. When UCH-L1 and GFAP were combined, increased sensitivity and specificity for diagnosing TBI was achieved.

Moreover, temporal serum profile analysis showed that UCH-L1 was able to distinguish between mild to moderate TBI patients versus control and performed best in the early post-injury period, peaking at 8 hours after injury and declining over 48 hours [128]. Finally, a recent study in children observed a step-wise increase of UCH-L1 in correlation of TBI severity (mild to severe) at an acute timepoint, immediately after hospitalization [142]. UCH-L1 was also able to predict outcome 6 month post injury in pediatric TBI. Thus, UCH-L1 is a promising candidate for mTBI biomarker research and is of great interest when combined with other potential markers, such as GFAP.

Axonal Injury

1. AlphaII-spectrin breakdown protein

AlphaII-spectrin breakdown protein is a major structural component of the cortical membrane cytoskeleton, being abundant in axons and presynaptic terminals [143]. Usually, during apoptotic and necrotic cell death cysteine proteases (calpain and caspase-3), cleave those components of the axonal cytoskeleton, leading to the formation of signature molecular weight products (spectrin breakdown products (SBDPs)) [144]. After TBI, calpains and caspases are hyperactivated, resulting in production of SBDPs and thus reflecting axonal damage [145]. One breakdown product is the 1176 residue α-spectrin N-terminal fragment (SNTF), which has been shown to increase measurably in the blood after TBI, including CT-negative mild TBI [137,146]. However, whilst SBDPs are highly

abundant in brain, they are not specific to neurons, and it has been shown that serum levels can reflect multiorgan damage [147].

2. Tau protein

Recently considerable attention has been drawn to the relationship between repeated mild traumatic brain injury (r-mTBI) and the subsequent occurrence of the degenerative neurologic disorder known as chronic traumatic encephalopathy (CTE), which was previously described as repeated head trauma leading to the development of dementia pugilistica (DP) [148]. Neuropathology of DP included neurofibrillary tangles in the absence of plaques, in contrast to the tangles and plaques seen in Alzheimer's disease (AD). However, not much is known about risk factors contributing to the subsequent development of CTE after r-mTBI. Clinical evidence comes from numerous studies, investigated mainly in boxers, but also in recent years in athletes and military personnel [149]. Before the term CTE was defined, Roberts and colleagues suggested that at least 17% of patients (boxers) with repetitive head trauma develop a neurological disorder [150]. Although it is not clear yet if CTE is a true tauopathy (only caused by tau), neuropathological features of CTE include widespread deposition of hyper-phosphorylated tau (phospho-tau/p-tau) as neurofibrillary tangles (NFTs) [15]. Multiple clinical reports have observed this accumulation of NFTs in the brains of athletes who sustained several concussions (reviewed by [151]). Furthermore, in preclinical mouse studies an increase in p-tau immunoreactivity in response to repetitive mTBI, but not to single mTBI has been observed [152]. Presence and levels of hyperphosphorylated tau isoforms in CSF of TBI patients have not been examined [136]. However, attention has been

brought to total tau, which has been examined as a biomarker of axonal injury in CSF and blood [136,153].

Elevated levels of total tau were observed in the CSF of patients with severe TBI, peaking 1 week post injury [154]. In another study increased CSF tau levels after severe TBI were correlated with clinical outcome [155]. In blood, elevated levels of total tau have been found to correlate with neurological outcome in resuscitated hypoxic brain injury from cardiac arrest patients [156]. Increased levels were also observed in plasma from patients with Alzheimer's disease [157], in plasma of Olympic boxers with head injury [158] and in concussed ice hockey players 6 days after the injury [159]. Furthermore, elevation in total tau concentration in blood was observed in military personnel who reported multiple TBIs [160]. Finally a recent study by Bogoslovsky et. al investigated a population of patients that consisted of 67% complicated mild TBI (cmTBI; patients with complicated mTBI show intracranial trauma-related abnormalities on a CT scan) with the remaining being diagnosed as moderate to severe TBI [129]. Peripheral tau, analyzed at 24hours, 30 days and 90 days post injury, was increased up to the latest time-point and associated with increasing severity and number of injury, as well as outcome of symptoms. Therefore, tau shows potential as a specific biomarker for the acute and sub-acute phase of TBI pathology. Furthermore, a cleaved form of tau (c-tau) has been proposed as a potential biomarker of CNS injury. After TBI, tau is proteolytically cleaved and can pass through the blood–brain barrier into the bloodstream when the nerves are injured. For example, increased c-tau levels measured in the CSF of severe TBI patients were a predictor of clinical outcome. Yet, when measured in the periphery this correlation could not be confirmed [161].

3. Neurofilaments (NF)

Neurofilaments are heteropolymeric components of the neuron cytoskeleton, consisting of three chains, namely light (L), medium (M) and heavy (H) [162]. In severe TBI cases, high levels of phosphorylated NF-H have been found in ventricular CSF [137]. Moreover, elevated levels of phosphorylated NF-L were observed in the CSF of boxers with acute mTBI, peaking 4-10 days after injury [163].

4. Amyloid β42 (Aβ42)

Aβ42 is widely known for its role in Alzheimer's Disease [164], yet studies started to elucidate its connection to TBI. After injury, Aβ42 is subsequently released from the damaged axons into the surrounding tissue, resulting in plaque formation [165]. An early study demonstrated diffuse Aβ42 pathology in the brains of 7 cases after fatal injury within 6 hours to 7 days [166]. Another post mortem study observed Aβ42 deposition in the brains of 39 long-term survivors (up to 47 years), who had sustained a single moderate to severe head injury [167]. Furthermore, Aβ42 was increased in the CSF in patients with severe TBI for up to 1 week [168,169]. A recent study by Bogoslovsky and colleagues showed that Aβ42 levels in plasma of complicated mild TBI patients were increased up to 90 days post injury [129].

Other potential CSF and blood biomarkers have been studied and extensively reviewed for TBI, such as myelin basic protein and the CSF:serum albumin ratio [136], however they lack the desired sensitivity and therefore all suggested molecules have

failed to gain FDA approval for biomarker testing. Additionally the lack of a "gold standard" diagnostic measure (imaging or biochemical) hinders further development of biomarkers, as new candidates have no standard against which specificity and sensitivity can be compared. So far only one company (Banyan Biomarkers) has entered into development agreement (a multi-year joint agreement with Royal Philips) to develop and commercialize a blood test to detect and evaluate acute mild TBI using Banyan UCH-L1™ and Banyan GFAP™ (http://banyanbio.com/media-news.html). Whilst this does not address the issue of chronic TBI, this could enable objective diagnosis of mTBI in the acute and subacute stages after injury.

1.5b PTSD biomarker research

PTSD biomarker research is limited due to the varying diagnostic procedures utilized and the heterogeneity of traumatic events to which PTSD patients were exposed. Additionally, differences in the study design and type of controls included in the investigation further challenges biomarker discovery and these variations make it difficult to perform a comparison between studies and their principal conclusions. As described above, PTSD is currently assessed through self-reports, clinical interviews and history documented by trained professionals. Self-report can always lead to insufficient information if a patient doesn't disclose all relevant information, especially in large scale screening processes, where a clinician is not always available to make a diagnosis. The difficulty in assessing and distinguishing co-morbidities adds to the issue of an objective diagnosis. As such an objective biomarker (or panel of biomarkers) would be a useful screening tool to detect PTSD in patients, who have difficulties describing their situation

and symptoms, and might also suggest treatments and facilitate evaluation of treatment responses [170]. Ideally, a large group of PTSD patients should be compared to a group of healthy controls (matched for age and sex) that were exposed to comparable traumatic events. These conditions are best achieved in military cohorts who can often have similar demographics and receive somewhat similar exposures. Although progress has been made in the characterization of PTSD pathophysiology and various studies have shown that genetic background [171], endocrine factors [172] and neurotransmitter systems [173] all modulate PTSD outcome and susceptibility, unfortunately, to date, there are still no generally accepted PTSD biomarkers in clinical use [174]. Consequently the potential PTSD biomarkers currently under investigation typically relate to these pathophysiological aspects of the disorder. The candidates, which have received the most attention are described below:

Potential biomarkers of PTSD include the consequences of enhanced HPA negative feedback in PTSD, which leads to increased peripheral cortisol and CSF CRH levels, as mentioned in the pathophysiology of PTSD section, although studies have shown confounding results [175–179]. It is also important to mark that cortisol levels have been shown to be influenced by different kinds of stress, diet, smoking, alcohol, sleep, exercise as well as changes in diurnal cycle [180,181]. Cortisol levels have also been implemented as a biomarker for patients with major depressive disorder [182], lacking specificity for PTSD. Moreover, reduced hormone binding potential of the GR receptor, as well as enhanced sensitivity to glucocorticoids has been observed in peripheral blood mononuclear cells (PBMCs) of PTSD patients [183,184].

In whole blood, gene expression analysis identified that expression of the GR-

regulatory gene FKBP5 was reduced in PTSD subjects [93]. However, FKBP5 has also been associated with mood disorders, such as major depression, and has been suggested as a biomarker for non-psychiatric disorders, for instance cancer [185,186].

Brain-derived neurotrophic factor, as mentioned above, plays an important role in proliferation, survival, and differentiation of nerve cells [187]. Multiple studies have evaluated BDNF as a biomarker for PTSD. For example, in plasma, reduced levels of BDNF were observed in PTSD subjects. Moreover, BDNF was found to correlate with PTSD severity over 6 months in survivors of motor vehicle accidents [188,189]. Although studies in general confirm findings of lower BDNF levels in individuals with PTSD, reduced BDNF has been associated with the pathogenesis of several neuropsychiatric disorders (reviewed by [190] such as major depressive disorder (MDD) [191]. As such BDNF lacks diagnostic specificity, underscoring the shared common pathophysiological mechanisms and comorbidity [192]

Genetic risk conferred by 5-HTTLPR in PTSD was discussed above, but in addition platelet 5-HT concentration has been associated with this condition, though as with many of these observations the findings are controversial. One study showed decreased levels of platelet 5-HT in veterans with PTSD compared to controls [193], whereas increased levels were observed in suicidal patients with PTSD [194].

For Neuropeptide Y, the observation of lower levels in the CSF of veterans with PTSD, could not be confirmed in PTSD survivors of motor vehicle accidents [195,196].

In conclusion, extensive efforts have been made to identify objective biomarkers of PTSD. This includes systems involving the HPA-axis, the neuroendocrine/metabolic

systems, neurotransmission, the sympathetic nervous system and others. Unfortunately to date, no such biomarkers have been proven reliable mainly due to the heterogeneity in PTSD symptom presentations and common comorbidity of PTSD. As such more work is needed.

1.6 Gulf War Illness

Posttraumatic stress disorder and TBI are not the only debilitating illnesses with high prevalence in military populations. Gulf War Illness (GWI) is a chronic multisymptom illness, which affects approximately one quarter of the 700,000 US veterans and one fifth of the 53,000 U.K. soldiers who were deployed in 1990-91 to the Persian Gulf [197–201]. Gulf War Illness is unique to the 1990– 91 deployment and distinguishes itself through a multiplicity and heterogeneity of symptoms persistent over decades observed in GW veterans, with no such illness being reported in any other military campaign, indicating that GWI etiology cannot solely be attributed to combat-related stress [202,203].

Veterans exhibiting GWI experience health issues such as, fatigue, idiopathic pain, gastrointestinal problems, muscle pain, and CNS-based symptoms, with memory problems being one of the most reported concerns [200,204,205].

Although it has been over 25 years since the Gulf War no effective treatment have been identified, and almost no veteran has recovered from this debilitating illness [197,206,207]. Furthermore, although the GWI patient population is discrete, the increased risk of neurodegeneration that appears to results from overexposure and/or chronic exposure to pesticides and insect repellents use has implications and relevance to other populations [208].

1.6.1 Clinical diagnosis of GWI

Clinical diagnosis of GWI relays on a symptom system cluster defined first by the Centers for Disease Control and Prevention (CDC) [198], which later was expanded by the Kansas criteria [200]. In order to receive a positive GWI diagnosis, Gulf War veterans must experience three out of six of the following symptoms: 1) fatigue/sleep problems, 2) somatic pain, 3) neurologic/cognitive/mood symptoms, 4) gastrointestinal symptoms, 5) respiratory symptoms, and 6) skin abnormalities [198,200]. However, due to the complexity of the condition and the lack of objective biomarkers, not all Gulf War veterans that suffer from symptoms that are consistent with GWI, are being diagnosed [209].

1.6.2 Pathophysiology of GWI

The Research Advisory Committee on GWI has named exposure to the anti-nerve gas agent pyridostigmine bromide (PB), the pesticide permethrin (PER) and the insect repellant N, N-Diethyl-metatoluamide (DEET) as key contributors to the GWI etiology [197]. Pyridostigmine Bromide is an acetylcholinesterase inhibitor (ACHEi), which was used by the soldiers as prophylactic agents against various nerve gases (e.g. sarin, mustard gas and soman). The carbamate compound PB reversibly inhibits the enzyme acetylcholinesterase (AChE), thus keeping nerve gas agents from degrading it. PER is a type 1 synthetic pyrethroid insecticide that function as a neurotoxin, affecting neuron membranes by prolonging sodium channel activation [210–213]. DEET, which is applied to skin or clothing, repels insects so that they are not attracted by odors produced by humans [214]. Others exposures have also been suggested to add to GWI symptomology, such as

depleted uranium and various vaccinations (e.g. against anthrax) [197,202,203,215–217].

It has been discussed that as a result of these exposures GW veterans exhibit alterations in HPA function, which has also been shown to affect veterans with PTSD (described above). Alterations include increased suppression of ACTH and cortisol in response to DEX [218–220]. Furthermore, impaired immune response has been suggested as a consequence of GW agent exposure [221]. GWI patients usually show altered expression in pro-and anti-inflammatory cytokines in peripheral immune cells although unity of expression patterns has not been achieved, most likely due to heterogeneity of symptoms and chosen cohorts. However, studies have shown that immune system alterations are especially pronounced in GWI patients during exercise challenge compared to controls. [222–227]

Other pathology has been observed in GW veterans and animal models of GWI such as CNS involvement through reduced volume and hypometabolism in the hippocampi of GWI veterans compared to controls [197], as well as lipid [228,229] and mitochondrial dysfunction [230–232]. Although much progress has been made in the past decade the underlying mechanisms are still poorly understand and are under intense investigation.

As for TBI and PTSD, it is likely that in GW veterans, genetic vulnerabilities to chemicals play a role in the post-deployment development of GWI [233]. However, research is limited and only one study has identified genetic influence on GWI presentation – this being variants of the enzyme butyrylcholinesterase (BChE) [234]. As mentioned above, PB, as well as organophosphates interact with circulating enzymes in humans, including the two cholinesterases - butyrylcholinesterase (BChE) and AChE, Whilst inhibition of

AChE interferes with the breakdown of the neurotransmitter acetylcholine, BChE has been shown to protect from the adverse effects of chemicals by acting as a scavenger. BChE binds chemicals molecule-for-molecule, thereby protecting circulating levels of AChE [235]. Besides the most common gene variant "U", multiple minor variants within the BChE gene have shown to be associated with lower enzyme activity levels and/or binding affinity for AChE-inhibiting compounds, making subjects carrying those variants more sensitive to different chemicals [235]. A GWI case-control study revealed that a subgroup of Gulf War veterans who had the less common (less enzymatically active) genetic variants were at significantly higher risk for GWI if they took PB pills during the 1990-91 deployment [234].

1.6.3 Biomarker research in GWI

To date no biomarker or biomarker panel is used in the clinical assessment of GWI. The persistent and heterogeneous presentation has made it not only difficult to identify a possible biomarker, but has also confounded comparison between clinical studies, as recruitment of veterans with the same symptom cluster is not easy to achieve. However, a recent blood biomarker study showed higher counts of lymphocytes, monocytes, neutrophils and platelets in GWI, as well as identified several proteins associated with inflammation to be differently expressed in patients versus healthy Gulf War veterans [236]. Although this first study requires further confirmation it holds potential.

1.7 The periphery as a source for biomarkers and the importance of translational research

As mentioned above in section 1.5, molecular biomarkers for neurological conditions mostly come from brain tissues, CSF and blood. Depending on whether samples are collected from animal models or humans certain advantages and disadvantages can be found for each source. Table 1.4 summarizes the value of each biomarker origin (adapted from [237]).

Sample host origin	Source of sample	Obtainable amount	Availability	Biomarker signal to noise
Human	Brain tissue	Low	Low	High
	CSF	Medium	Medium	Medium to low
	Blood	High	High	Low
Animal	Brain tissue	High	High	High
	CSF	Low	Low	Medium
	Blood	Medium	Medium	Low

Table 1-4 Advantages and disadvantages of sample sources in animal and humans

In animals, brain samples can be obtained in a controlled laboratory environment, at any time of choice post-exposure to the insult or trauma, whereas in humans samples can only come from biopsies (sometimes occurring in severe TBI cases) or from deceased patients, leading to the introduction of post mortem artifacts.

Parenchymal cells of the brain and spinal cord are surrounded by the interstitial fluid (ISF), whereas spaces within and around the CNS are filled by the cerebrospinal fluid (CSF). To guarantee proper brain cell function and survival the ISF provides the

environment within with water and solutes. Furthermore, waste products need to be removed. The CSF serves a number of functions, which include buoyancy, protection, chemical stability, waste removal. CSF is also important in removing protein and other debris during and after brain ischemia[238].

Circulation of these fluids is essential for proper brain cells functioning, which is regulated by different barriers that prevent free exchange between brain interstitium, CNS and blood. The structures associated in the process can be found below in Figure 1-5 and include the choroid plexus, the arachnoid villi, as well as glial, pia and endothelial barriers.

Most of CSF is secreted by the choroid plexus into the ventricular cavities, whereas the BBB generally produces interstitial fluid (ISF), which drains into CSF via bulk flow. It is traditionally known that after production by the choroid plexus, the CSF then circulates in a bulk flow manner, unidirectional through the ventricles and the subarachnoid spaces, to then be passively absorbed into the venous blood at the level of arachnoid villi[239]. It has been shown that on the level of the arachnoid villi a pressure-sensitive vacuolation cycle of one-way pores, allow for the transcellular bulk transport of fluid, with molecules passing through up to the size of erythrocytes.

However, newer experiments support the hypothesis by Klarica and Oreskovic, which shows that CSF circulation is not unidirectional, but rather there are cardiac cycle-dependent systolic-diastolic to-and-fro cranio-spinal CSF movements via physiological oscillations of arterial and venous blood during cranio-spinal blood circulation, respiratory activity, and body activity and posture[240]. Another portions of CSF (40–48% of total volume may be absorbed by extracranial lymphatics [241]. Additionally, studies

have shown ISF drainage into the lymphatic systems.

The CSF also circulates along the Virchow–Robin spaces (VRS), which represent the space surrounding vessels penetrating into the parenchyma. Glial and pial barrier membranes that surround the VRS control the bidirectional fluid exchange between the VRS and the brain extracellular space.

The BBB and its tight junctions have a low permeability for polar solutes; fluid secretion is regulated by Na+/K+-ATPase-depended ion channels and transporters on the brain side of the barrier. The astrocytes on the BBB play an important role in brain water homeostasis and research has shown the existence of water transporting pores (i.e. the aquaporins), which are localized in the end feet[242].

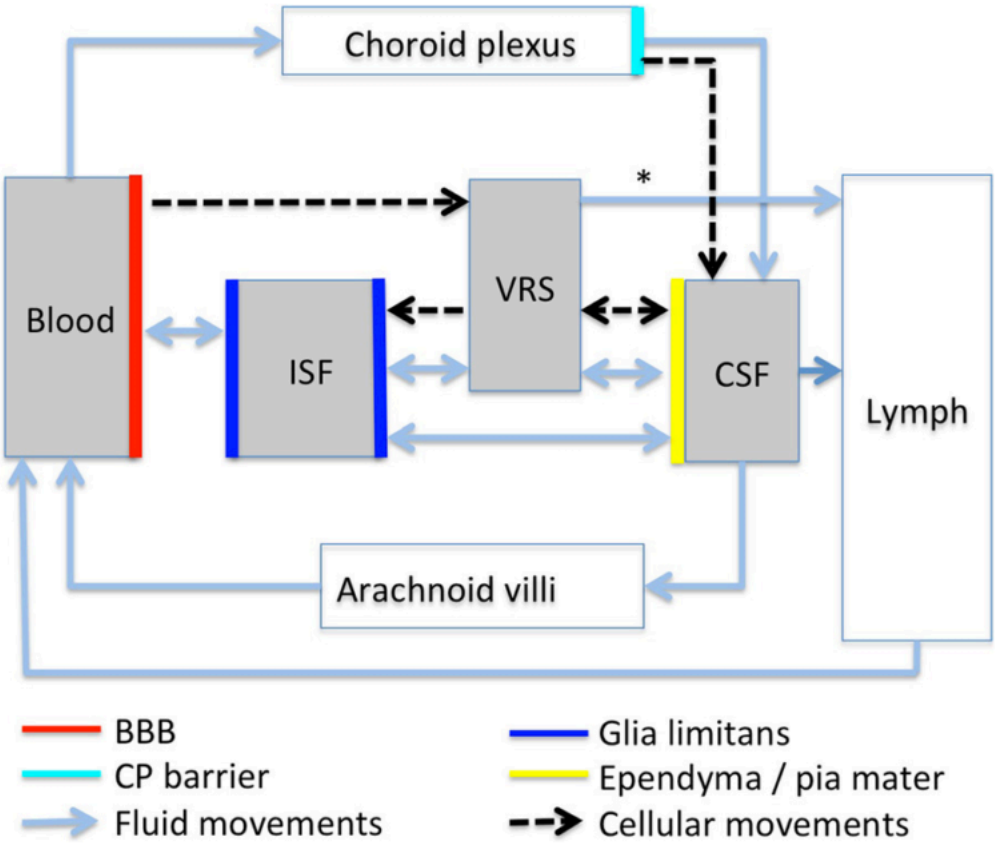


Figure 1-5 Overview of the CSF "circulation": Different barriers limit fluid and cellular movement across the brain compartments, which include blood, interstitial fluid, the Virchow Robin space and the cerebrospinal fluid space comprising the cerebral ventricles, basal cisterns and cortical subarachnoid space. The choroid plexus (CP), glial and endothelial barriers control fluid exchange via aquaporins and other transporters. Net CSF production rate is surpassed by the fluid exchange rates generated the bi-directional flow at the glial, endothelial, and pial barrier. Inflammatory cells from the blood enter the brain through the VRS or CP, whereas brain water is mostly drained into the cervical lymphatics from the VRS and from the CSF space. Between the blood and the CSF compartment the CP is the only direct connection. Whilst fluid movements into and out of the VRS is driven by respiratory and cardiac pressure pulsations, movements at the barriers is depended on osmotic and hydrostatic gradients or by active transporter processes (picture adapted with permission from Brinker et al. 2014[243].

For CSF, the collectable amount in rodents is small and contamination with blood is an unwanted possibility if puncture technique is not done properly. In humans, CSF collection is routine and larger volumes can be collected, but the technique is invasive.

Furthermore contamination of the CSF with blood is possible, which can falsify scientific findings.

In humans and animals, blood is the preferred matrix for analysis of biomarkers, owing to their easy and cost effective collection. Changes in the brain can be expected to result in altered protein or lipid levels, that will eventually be reflected in the blood (through the CSF) [111,244]. The identification of peripheral blood biomarkers for mTBI, PTSD and GWI would provide many advantages. Expensive imaging techniques fail to detect the majority of mTBI injuries, PTSD and GWI patients and are much more costly to perform than analysis of easily accessible blood samples.

Due to the clinical presentation of mTBI, PTSD and GWI, more precisely their heterogeneity in regards to injury severity, physical mechanisms, pathobiology, multiple impact, nature of traumatic exposure etc., it is difficult to not only identify a specific and sensitive biomarker, but also develop relevant animal models. Basic research scientists and clinicians often work in separate settings, leading to a gap in informational exchange. However, validation of animal models and translational studies are essential to diagnose, prognose and further investigate treatment outcome. It is of importance that underlying mechanisms of the mentioned conditions are studied in appropriate animal models that reflect characteristics of the clinical presentations seen in patients. Animal models present the possibility of investigation of peripheral biomarkers in combination with changes in the brain, at a range of timepoints post-exposure, and results from human blood analyses can then be "mapped" against the profiles from preclinical studies.

1.8 Hypothesis & Synopsis of following chapters

Given the likely critical role of brain changes in proteins and lipids in the pathogenesis of mTBI, PTSD and GWI, I hypothesize that protein and lipid biomarkers exist in the plasma and can be detected at chronic stages. These biomarkers could be combined in a panel that can assist in diagnosis of these conditions post exposure.
In this study, we used two "omic" approaches, measured via mass spectrometry (MS) to identify changes in plasma in humans and animal models.

In Chapter 2 we investigated protein changes (*proteomics*) among a cohort of active-duty military soldiers, which identified the protein Leucine-rich alpha-2-glycoprotein 1 (LRG1) to be chronically significant different. In Chapter 3 we identified differences in lipid levels (*lipidomics*). Our findings, which indicated a strong lipid signal, were then translated to a mouse model of mild TBI. Additionally, LRG1 was measured in these animals (Chapter 4). In this model we were able to observe temporal profiles of lipid changes ranging from acute (24 hours) to chronic (24month) stages post insult to show that our model is relevant to human mTBI patients and as such is well fitted for translational studies. In Chapter 5 we were able to investigate the debilitating condition of GWI. In this chapter we used lipidomics on a veteran population and compared our findings to two rodent models (mouse and rat) to verify findings and the power our analytical tools. In Chapter 6 I discussed the overall findings in this thesis, which showed that there are chronic biological changes in military relevant conditions that could be used to track illness and help identify potential therapeutic targets.

Chapter 2 Proteomic discovery phase and validation of a protein biomarker from a military cohort study with soldiers exhibiting mild traumatic brain injury and/or posttraumatic stress disorder

2.0 Summary

Traumatic brain injury (TBI) is a major problem for military and civilian populations, with long-term sequelae including neurological and neuropsychological dysfunction. Furthermore, in military service members and veterans there is a high prevalence of comorbid TBI and posttraumatic stress disorder (PTSD) due to the inherent risk of psychological trauma associated with combat. An objective panel of biomarkers for TBI and PTSD would enable triage during acute care and appropriate medical management, and may indicate ongoing pathogenic processes, provide guidance in therapeutic development, and could be used to monitor outcome and response to treatment. The special need for biomarker discovery at late stages post exposure comes from the fact that both mTBI and PTSD are chronic conditions, the difficulties in diagnosing mTBI or PTSD and the issue that these conditions are not typically identified or assessed in the acute phase. Therefore, in this study we investigated proteomic plasma profiles of 120 active duty soldiers categorized with mild TBI, PTSD or TBI+PTSD, as well as healthy controls, via liquid chromatography mass spectrometry (LCMS) in order to identify candidates for chronic biomarkers of these conditions. Data analysis revealed three

significant proteins to be differently expressed between diagnostic groups before post-hoc analysis. Although no protein passed multiple test correction following LCMS analysis, we used ELISA to further investigate the protein LRG1, which had shown the most significant change. The antibody-based approach revealed a significant increase of LRG1 by 2.8 fold in the TBI+PTSD group compared to controls.

2.1 Introduction

Although extensive research has uncovered major underlying biological features of TBI and PTSD, the question of an objective measurement for both conditions remains. While progress has been made in the acute setting of TBI, diagnostic tools are needed for later timepoints post injury.

Plasma is widely accepted for use in clinical testing, and the fact that it is easily obtainable, being non-invasive and cost effective, makes it a favorable sample to study. It contains molecule,s which may be relevant for prediction, diagnosis or cause and effects of neurological disorders. Due to the increasing need to identify and quantify proteins in complex biological sample matrices, such as plasma, with higher sensitivity and specificity, the platform of liquid chromatography mass spectrometry (LCMS) has evolved.

MS is a powerful analytical tool for protein research, as it can measure the relative or absolute abundance of a protein or proteins among biological samples. One major strategy of global proteomics includes an unbiased discovery oriented approach, which studies differential global expression of the proteome across varied conditions for biomarker purposes or to identify new proteins and pathways related to the condition of interest. Tandem mass spectrometry (the use of successive rounds of mass analysis and fragmentation on a single analyte, herein referred to as "MS/MS" or "MS^2"), has been the dominant driver for the generation of proteomic data [245,246]. Protein samples are digested into peptides, typically with trypsin (see below). The peptide mixture is then separated via liquid chromatography (LC) prior to introduction into the MS instrument,

which generates data pertaining to the intact mass of the peptide (precursor, or "parent" mass). This is followed by a fragmentation event within the MS whereupon the peptides are dissociated along the amino acid backbone, thus providing a sequence-informative spectrum, which is later paired with the parent mass during data analysis. These fragment ("product") spectra are referred to as MS/MS. Depending on the complexity of a sample, a modern mass spectrometer may generate tens of thousands of parent/fragment ion spectra within a single hour of runtime.

Although plasma is an easily obtainable biofluid that contains a wealth of potential diagnostic information about the subject, the primary obstacle for obtaining deep proteomic coverage is the occurrence in blood of several highly-abundant proteins, such as serum albumin and immunoglobulins, together constituting more than 85% of the total protein content. These proteins mask lower abundance proteins, which could be potential candidates for biomarkers. In order to avoid this masking effect, highly abundant proteins must first be depleted from the sample [247,248] (see methods section below). The remaining proteins are then digested with one or more protease, such as trypsin. Trypsin is the most routinely-applied protease for this purpose owing to (1) fairly consistent cleavage specificity (which yields peptides with at least one defined terminal amino acid (a useful piece of information during database searches), (2) the fact that the resultant tryptic peptides have a basic terminus, which facilitates fragmentation, and (3) the charge state of most tryptic peptides being amenable to fragmentation in most modern mass spectrometers relative to other proteases which cleave less frequently (and hence, have larger charge states).

In MS, relative quantification of proteins in samples can be achieved via a variety of approaches, summarized in Appendix, Chapter 2; Section 1 Table 1. In this work we have chosen to use isobaric tagging of peptides prior to analysis, primarily as a means to accelerate the workflow, and also to improve the standardization of the LCMS analysis. The multiplexing capacity of isobaric labeling techniques, such as the tandem mass tag (TMT) 10-plex approach, allows for pooling of up to 10 samples, thereby reducing the number of LCMS analyses and instrument time required [249,250]. It also lowers the "missing value problem" that is inherent to data-dependent acquisition (DDA) mass spectrometry, commonly used in proteomic discovery phase experiments. This problem results from the semi-random nature of precursor peptide selection in complex samples, and ultimately leads to under-sampling and inconsistent data when matching peptide identifications from multiple LCMS data sets [251].

Researchers conducting proteomic studies have previously used proteolytic digestion followed by isobaric labeling in humans and mouse models of TBI and/or PTSD. For example, Wishart et al. analyzed pooled iTRAQ (isobaric tagging for relative and absolute quantitation) labeled samples from brain tissue synapse-enriched brain tissue of mice to detect differential synaptic protein expression in TBI 72hrs after injury [252]. This resulted in a refined profile comprised of 93 proteins.

Our group has previously performed an iTRAQ based proteomic analysis to investigate plasma proteomic profiles in a mouse model of severe and relatively mild TBI at 24 h, 1 month, or 3 months post-injury [253]. In that study, results generated by MS analysis were further investigated for their function using Ingenuity Pathway Analysis (IPA) software. We observed injury dependent changes in plasma proteins as well as temporal differences

post-injury in changing plasma protein levels between mild and severe TBI with acute elevation of certain proteins in mild TBI possibly indicating a reparatory process. Furthermore, the work was carried out in apolipoprotein E (APOE) 3 and APOE4 transgenic mice, which demonstrate relatively favorable and unfavorable outcomes respectively following TBI. Thus the data analysis included identification of proteins that were regulated differentially as a response to TBI in the different genotypes and thus may be relevant as prognostic biomarkers.

A study in 11 severely injured TBI patients identified 31 serum candidate biomarkers, including serum amyloid A, c-reactive protein and retinol binding protein 4, 24–36 hours post-injury via LC-MS/MS [254].

There are a few published studies that utilize isobaric tagging proteomic approaches for PTSD in animal models. For example, Hennigsen et al. used a rat model of chronic mild stress and iTRAQ to identify 73 differently expressed proteins in the hippocampus [255]. In a related LCMS based field – metabolomics - researchers found metabolites involved in processes of (neuro-) inflammation, auto-immune reactions, oxidative stress, energy metabolism, and biological aging serum samples of PTSD patients and controls [256].

Although these studies have shown the potential of proteomic data generation, more investigation is required, as well as replication in other cohorts. Moreover, TBI studies have been performed in acute and sub-acute settings only [254]. As such the identified proteome differently expressed in plasma, most likely represents the leakage through the disruption of the blood brain barrier, which, once repaired, will most likely lose the signal of those identified proteins. Analysis of acute samples is of importance in hospital

settings, however especially in case of military active duty soldiers, immediate care is often not present. In that case the usual acute biomarkers might no longer be present by the time a soldier gets admitted. This emphasizes the importance of identifying proteins that are differentially regulated at chronic timepoints. Furthermore, as mentioned in the Introductory Chapter 1, 83% of sustained TBI's are mild, underlining the need for biomarker discovery for those cases.

My objective was to examine the alteration in global expression of proteins in the plasma of mild TBI and PTSD subjects in comparison with healthy controls. The TMT approach was utilized to analyze albumin-depleted plasma samples to reveal candidate biomarkers. TMT labeled groups obtained from digests of the prepared plasma samples from healthy controls, as well as subjects with a history of mild TBI, chronic PTSD and mild TBI+PTSD, were analyzed by LCMS. It was expected that studying plasma proteins in these chronic samples using discovery-based proteomics could identify potential new protein biomarkers that persist in the periphery well after the initial insult(s).

2.2. Methods

Research participant selection

Pre-deployment data from 120 active-duty U.S. soldiers, aged 18 to 49 years old, were collected within 30 days prior to a 12 -18 month deployment to the Middle East for Operation Iraqi Freedom (OIF)/Operation Enduring Freedom (OEF). Participation was voluntary and the study was carried out in collaboration with Dr. (Major) Michael Dretsch and his team at the United States Army Aeromedical Research Laboratory, (USAARL) in Alabama. There were no inclusion/exclusion criteria because all soldiers were deemed medically fit for deployment (i.e., physical and psychiatric screening) through the deployment medical screening.

Procedures

The study was approved by the Institutional Review Board (IRB) at Headquarters U.S. Army Medical Research and Materiel Command, Fort Detrick, MD. HQ USAMRMC IRB and the protocol and procedures were carried out in accordance with the latest version of the Declaration of Helsinki. Soldiers were given the opportunity to voluntarily participate after receiving a study brief by the Principal Investigator (Dr. Dretsch) at a designated facility on the military installation. Data were collected from two brigade combat teams deploying to Iraq and Afghanistan (active-duty US Army soldiers from the 2nd Brigade, at Fort Riley, KS. and the Ohio National Guard stationed at Camp Shelby, Mississippi). An IRB approved informed consent was provided to each potential participant in accordance with the International Conference on Harmonization (ICH)

guidelines and a signed consent was obtained from all participants in the presence of an ombudsperson. The volunteers were then escorted to a separate classroom for computerized testing and to the phlebotomy stations where several tubes of blood were drawn for genotyping and pre-processing for omic analyses. Standard operating procedures (SOPs) were employed for recruitment and sample processing for all individuals (controls, TBI, PTSD and TBI+PTSD subjects) so that they were identical. Briefly, samples were processed and frozen immediately after collection. For whole blood, a 1 ml aliquot was transferred from the vacutainer tube a 2.0ml Eppendorf tube. In order to minimize pre-analytical artifacts Ethylenediaminetetraacetic acid (EDTA) tubes were used for plasma collection. Plasma was then centrifuged for 10 minutes at 3,200 x g RCF at 4ºC before being added to protease inhibitors (antipain and pepstatin) in order to prevent protein degradation. Plasma was then aliquoted into Eppendorf tubes and samples were immediately stored in a dry ice freezer prior to transport to a -80°C medical specimen freezer at the end of each day.

Throughout the study technicians were blinded to the diagnostic status of the samples. Members of the Roskamp Institute staff were deployed to both sites during the recruitment periods in order to conduct and oversee all blood collection and processing according to our SOPs. Approval was attained from brigade commanders. Participants received a check for $50 (USD) for the blood draw.

Psychological tests and questionnaires **(the following described tests can be found in the appendix, Chapter 2; Section 2):**

Screening included basic demographics including: history of TBI (including military-associated injury and all other traumas), alcohol use, medical history, prior deployments, current medications, PTSD symptoms, and depression.

PTSD Checklist—Military Version (PCL-M)[257]*:* The PCL-M is a 17-item self-report measure of the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) symptoms of PTSD. The used cut-off score of ≥35 has been shown to have sensitivity and specificity for screening troops for PTSD is recommended by the National Center for PTSD[258].

The Life Events Checklist (LEC) [259]*:* The LEC is a 17-item self-report measure, screening for potentially traumatic events in a respondent's lifetime. Sixteen of the items assess exposure, which is known to potentially result in PTSD or distress; the remaining item assesses any other extraordinarily stressful event not captured in the first 16 items.

Brief Traumatic Brain Injury Screen (BTBIS) [260]*:* The BTBIS is a three-item assessment used by the Army to identify soldiers who may have sustained an mTBI. Subjects identify the mechanism (e.g., fragment, vehicular, blast, etc.), symptoms that occurred immediately after the injury, and current symptoms believed to be associated with an injury. A positive mTBI score required both the endorsement of an injury-related event

and, at minimum, an altered state of consciousness (e.g., being dazed, confused, or "seeing stars"; post-traumatic amnesia; loss of consciousness <20 min).

Zung Depression Scale (ZDS) [261]*:* The ZDS is a 20-item self-report depression rating scale. The ZDS produces scores ranging from 20 through 80; 20–44 for normal range, 45–59 for mildly depressed, 60–69 for moderately depressed, and 70 and above for severely depressed.

Zung Anxiety Scale (ZAS) [262]*:* This ZAS is a 20-item self-report anxiety rating scale. The ZAS has scores ranging from 20 through 80; 20–44 for normal range, 45–59 for mild-to-moderate anxiety, 60–74 for marked to severe anxiety, and 75–80 for extreme anxiety.

Alcohol Use Dependency Identification Test (AUDIT) [263]*:* This 10-item instrument is used to assess alcohol use. A score of 8 or more in men, and 7 or more in women, indicates possible hazardous or harmful alcohol use. A score of 20 or more suggests potential alcohol dependence.

Epworth Sleepiness Scale (ESS) [264]: This instrument asks the subject to rate his or her probability of falling asleep on a scale of increasing probability from 0 to 3 in eight different situations.

*Pittsburgh Sleep Quality Index (PSQI) (*Buysse et al. 1989*):* This 19-item instrument is used to measure sleep quality during the previous month and discriminates between good

and poor sleep. The PSQI assess several domains including subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleep medications, and daytime dysfunction.

Neurocognitive measures

Central Nervous System-Vital Signs (CNS-VS)[265]: The CNS-VS is a computerized neurocognitive assessment battery. The present study used five CNS-VS subtests (verbal memory, symbol digit coding, Stroop test, continuous performance test, and shifting attention test). The CNS-VS domain scores calculated were verbal memory, complex attention, reaction time, processing speed, cognitive flexibility, and executive functioning.

Plasma protein fractionation

For proteomic analysis, after lipid depletion through a non-denaturing solvent system, the dynamic range of protein abundance in plasma was first reduced through protein fractionation and albumin depletion followed by alkylation. Protein concentration for each sample was assessed before tryptic digestion, peptide labeling and offline high-pH reversed phase chromatography in order to decrease sample complexity at the peptide level. This was followed by LC MS/MS analysis. For proteomic experiments low-retention tips/tubes were used and all reagents were LCMS grade.

Non-denaturing delipidation:

Equal amounts of sample from each subject (150µl) were centrifugally delipidated according to van Eyk, and the partially clarified plasma was transferred to a new tube. Each sample was then diluted with 400µl of chilled phosphate-buffered saline (PBS). 500µl of a non-denaturing solvent system [2:3 n-butanol : DIPE (diisopropyl ether) [266]] was added for further delipidation of plasma. After centrifugation, the upper (lipid-containing) phase was removed and the lower (protein-containing) phase was used for protein fractionation.

Albumin depletion and protein fractionation:

Albumin depletion was carried out by addition of concentrated ethanol to a final concentration 42% v/v with shaking to avoid sample variability due to localized concentrations of EtOH that exceed 42% v/v. The samples were then incubated at 4ºC for one hour, followed by centrifugation to collect an albumin-depleted fraction. The resultant pellet was resuspended in 25mM Triethylammonium Bicarbonate (TEAB), pH = 8.0 and 0.5% sodium deoxycholate (SDC). Protein concentrations for each sample were assessed using the bicinchoninic acid **(**BCA) assay (standards were 0, 5, 10, 20, and 30 µg of BSA per 50 µl of buffer) and prepared in triplicates. The color change of the BCA reagent was measured via absorbance at 562nm. BCA concentration data and sample integrity were verified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and staining. 10µg of the delipidated, albumin-depleted samples were separated on a 4-12.5% Nu-PAGE gel (Invitrogen) in MES (2-(N-morpholino) ethanesulfonic acid) buffer. Following electrophoresis, gels were fixed in 30% isopropanol, 2% H_3PO_4, followed by Sypro staining and imaging.

Tryptic digestion, internal standards, and Tandem Mass Tagging (TMT)

Following verification, equal amounts of sample from each subject (100µg) were de-salted using acetone precipitation and the pellets were re-suspended in 10µl of 10X reduction, alkylation, resuspension buffer (RB), containing 25mM TEAB, 5% SDC and 1mM tris(2-carboxyethyl)phosphine (TCEP) and 2mM chloroacetamide (CAM) for simultaneous sample reduction and alkylation. Samples were incubated for thirty minutes at 37ºC in darkness. Finally, each sample was digested by addition of 90µl 25mM TEAB supplemented with 0.5µg of porcine sequencing grade trypsin, and the small subunit (ss) of Rubisco (0.125ug/sample) as an internal standard [a plant protein with an amino acid sequence not present in the human proteome, ssRubisco will not interfere with identification of endogenous plasma proteins. Samples were digested overnight at 37ºC. In order to enable multiplex relative quantitation by mass spectrometry of ten different samples the Thermo Scientific TMT ^{TM} Mass Tag Labeling Kit (10plex) was used. The TMT reagents contain an amine-reactive group, which covalently binds to peptide N-termini or to lysine residues, a MS/MS reporter and mass normalizer (balancer) group. Each isobaric reagent contains an identical set of light and heavy isotopes that are uniquely divided between the balancer and reporter regions (Figure 2-1).

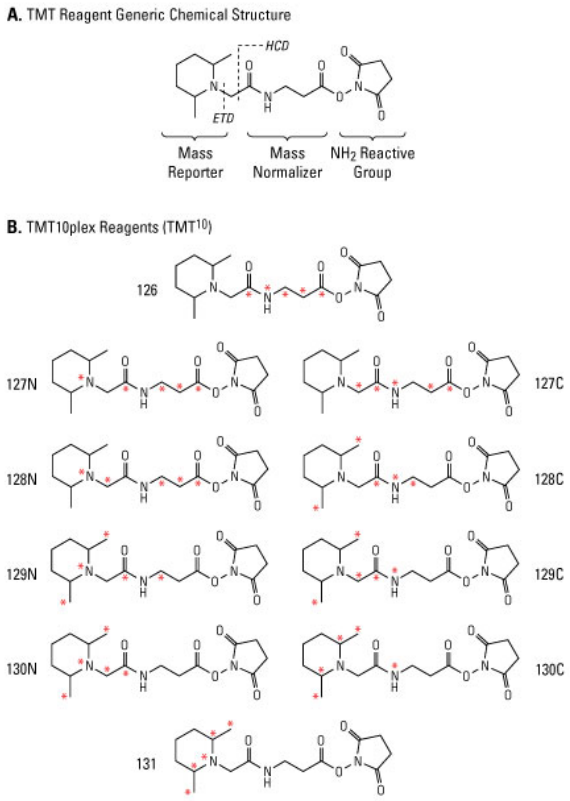


Figure 2-1 Chemical structures of the amine-reactive Tandem Mass Tag™ Reagents

A. Functional regions of the reagent structure including MS/MS fragmentation sites by higher energy collision dissociation (HCD) and electron transfer dissociation (ETD). B. Structures of the TMT10 plex reagent distinguishable through the 13C and 15N heavy isotope positions (shown in red asterisks). Picture permitted to use from Thermo Fisher.

Therefore, all reagents have an identical mass, while each reporter region produces a unique m/z reporter ion. Since each sample is derivatized with a different isotopic variant of an isobaric mass tag from a set, samples can be pooled and analyzed simultaneously in MS after being labeled (Figure 2-2).

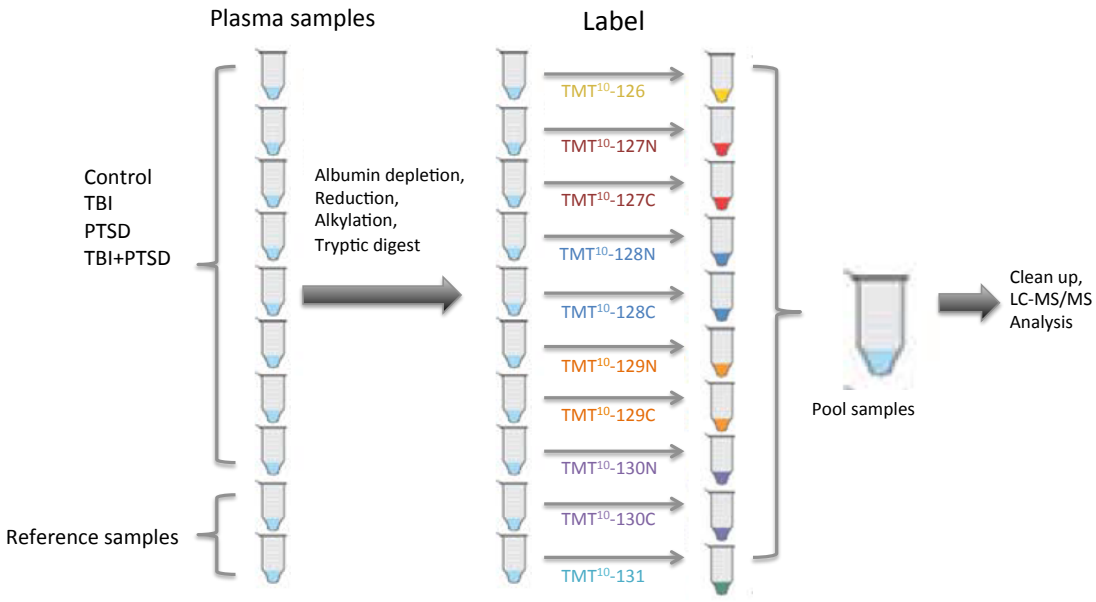


Figure 2-2 Example of TMT-based proteomic workflow, adapted from Thermo Fisher TMT-10plex system.

In the MS1 scan peptides labeled with isotopic variants of the tag appear as a single composite peak at the same m/z value with identical liquid chromatography (LC) retention time (Figure 2-3, top). The following MS/MS fragmentation generates from each modified precursor ion reporter ion peaks and peptide fragment ion peaks (Figure 2-3, bottom). Quantification is then achieved via correlations of the relative intensity of reporter ions to that of the peptide selected for MS/MS fragmentation. For peptide identification, fragment ion peaks are used which are observed at higher m/z. They are specific for peptide amino acid sequence that can be assigned to the proteins that they represent. Finally, TMT reagents label every tryptic peptide; therefore more than one peptide representing the same protein can be identified, which increases identification and quantification confidence.

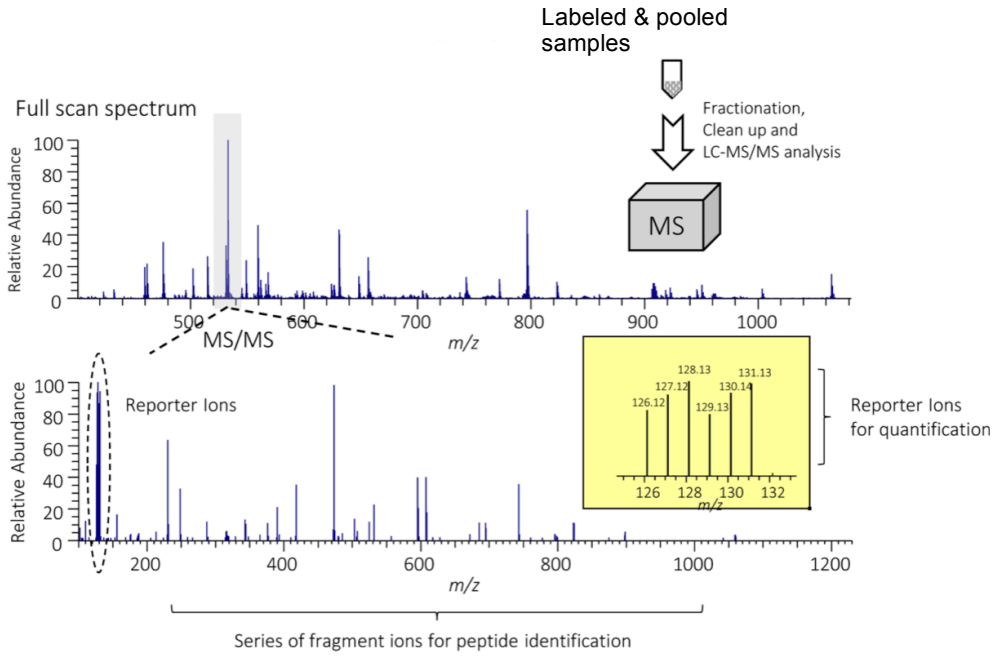


Figure 2-3 MS workflow of an isobaric labeling experiment. Following MS1, peptides with the same sequence from different samples appear as a single unresolved additive precursor ion. After MS/MS, the ten reporter ions (here only shown six) appear as distinct masses between m/z 126−131, and the remainder of the sequence-informative b- and y-ions remains as additive isobaric signals. Intensity of the reporter ion indicates the relative amount of peptide in the mixture that was labeled with the corresponding reagent. Used with permission from Rauniyar et. al. [267]

Participant samples (Controls, TBI, PTSD and TBI+PTSD) were labeled using TMT reagents 126−129 (N and C tags included), while the reference samples (pooled, depleted plasma from multiple healthy controls) were consistently labeled using TMT-130C and TMT-131. The experimenter was blinded to the diagnostic group. There were a total of 120 participant samples to analyze, and additionally two reference samples required per 10-plex, which led to a total of fifteen separate 10-plexes. Each TMT sample was resuspended in 20mM TEAB in acetonitrile (ACN) and then added to 10µg of dried peptide samples. Samples were incubated with their respective label for 1.5h in the dark

and then pooled (10 samples with 10 different labels) into one sample and 0.1% formic acid (FA) was added to stop the reactions. Labeled samples within each TMT 10-plex set were combined into one vial and processed according to the workflow outlined in Figure 2-2. Each 10-plex contained randomized samples, but was balanced in regards to number of controls, TBI, PTSD and TBI+PTSD subjects.

Offline high-pH reversed phase chromatography

In order to decrease sample complexity at the peptide level, a modified protocol adapted from Gilar [268] was used to perform two-dimensional high pH reverse phase spin column chromatography (HPRP) prior to LCMS analysis for each pooled 10-plex. Dried samples were re-suspended in 20mM Ammonium Formate (pH = 10) and applied to a C18 reversed phase Spin Column (Thermo). Acetonitrile (ACN) in binding buffer was added to the column with stepwise increments of 10% ACN, yielding 5 fractions (0, 10, 20, 30, and 40% acetonitrile in 20mM Ammonium Formate) for each sample. All samples were dried down and re-suspended in 10µl 0.1% FA in preparation for additional de-salting using C18 reversed phase ZipTips (Millipore) according to manufacturer's instructions.

LCMS/MS

To remove residual ACN following HPRP, the peptide fractions were again taken to dryness in a vacuum centrifuge. After being dried down again, samples were re-suspended in 0.1% FA and applied to a 0.075 x 150mm C18 (1.3µm particle size) Pepmap column (Dionex). Separation was carried out for over a 100-minute linear gradient from 2 to 30 % acetonitrile at 300nl/min. Mass spectrometry was

performed using a Q-Exactive Orbitrap (Thermo). The MS was run in data-dependent acquisition (DDA) mode, using a top-10 duty cycle, wherein one full scan spectrum was acquired, followed by ten MS/MS spectra. The scan ranges were 380 – 1250 m/z for full scans, and a minimum m/z of 100 for MS/MS scans (the maximum m/z of each MS^2 scan is derived from the precursor mass of the peptide being fragmented). Full scan MS resolution was set to 140,000 (FWHM, at m/z = 200) resolution and 35,000 resolution for Higher-energy C-trap dissociation (HCD) MS^2 spectra. The isolation width was set to 1.2 m/z and MS^2 reactions were performed at chromatographic peak apex. These settings were chosen as a compromise between quantitative accuracy (relies on statistically-reliable numbers of full scan spectra for peak integration) and sensitivity (number of peptides identified).

Proteomic Data Processing and Statistical Analysis

Samples were analyzed by LCMS/MS (Q-Exactive). PMi Preview software was used to survey the data files and if necessary add other modifications to the search criteria. Also, Preview results were used to choose the precursor and fragment ion mass tolerances (4 ppm, 0.02 Da, respectively). The Uniprot Human database (Downloaded march 2014) and common contaminant protein database provided by Byonic used in searches. The following settings were used to search the data using SEQUEST and BYONIC as the search algorithms; dynamic modifications; Oxidation / +15.995 Da (M), Deamidated / +0.984 Da (N, Q), Methyl / +14.016 Da (H, N, R) static modifications of TMT 10 plex / +229.163 Da (N-Terminus, K), Carbamidomethyl +57.021 (C). Only unique peptides were considered for quantification purposes. The peptide validator

feature of Proteome Discoverer 2.1 was used to set a false discovery rate (FDR) of 0.01. Quantitative analysis of the TMT experiments was performed simultaneously to protein identification using Proteome Discoverer software. Reporter ion quantification of HCD MS^2 spectra was enabled, and TMT-10 plex was set as the quantification method. The quantification values were extracted from the reporter ion intensities. The "Total Peptide Abundance" method was used to adjust for loading bias. Reference samples 130C and 131 were utilized to allow for cross set comparison. However, upon manual checks it was decided that channel 130C was not consistent across different plexes and was excluded from analysis. TMT channel 131 was used as a denominator to control abundance variation for each plex. Peptides identified in at least 8 of plexes were used in the quantitative analysis. Proteins (including the ones identified by a single peptide) were only included in subsequent analyses if they met the following requirement: the peptides that were assigned to them could not be assigned to other possible proteins. Proteins passing this cutoff value were exported to JMP (SAS) 8.0.2 for data cleaning and statistical analysis. After $\log_2$ transformation of the raw ion counts, each channel was divided with channel 131. The protein level abundance ratios were calculated according to formula 2.1 where p is a given unique peptide.

$$Protein = \frac{P1+P2+P3....+Pn}{P1+P2+P3....+Pn} \qquad \textit{Formula 2.1}$$

Finally, we used mixed model ANOVA where *Subject ID* was a random variable nested in *Plex no.* and *Category*, and where *Plex no.* was random and *Category* were

fixed effects, to identify proteins that were regulated across comparison groups (*Category*). Statistical analysis was performed using JMP 8.0.2 (SAS). The multiple testing correction as per Benjamini & Hochberg (B-H) [269] was applied to have a FDR of 5 percent. P values < 0.05 were considered statistically significant.

ELISA

The concentrations of human leucine-rich alpha-2 glycoprotein 1 (LRG1) and hemoglobin subunit beta (HBB) in plasma were determined using a competitive inhibition enzyme-linked immunoassay (LRG1 ELISA (MyBioSource.com) and HBB sandwich ELISA (LifeSpan BioSciences, Inc, WA) respectively), following manufacturer's instructions. Briefly, for LRG1, samples were added to 96-well plates that were coated with a Horseradish Peroxidase (HRP) conjugated LRG1. A competitive inhibition reaction was launched between LRG1 in samples and HRP-conjugated LRG1 with the pre-coated antibody specific for LRG1. The more the amount of LRG1 in the samples, the less antibody bound by HRP-conjugated LRG1. Samples were washed and a substrate was introduced that was converted by the enzyme attached to the detection antibody and color development was inversely related to the amount of LRG1 in the sample. The color development was stopped and absorbance of each well was quantified at a 450nm wavelength.

For HBB, samples were added to a 96-well plate that was coated with a target specific capture antibody. A biotin-conjugated detection antibody was added followed by a wash step and an HRP conjugate, which bound to the biotin. Unbound HRP conjugate was washed away and a 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate added which reacted with the HRP enzyme resulting in color development. The color development

was stopped and absorbance of each well was quantified at a 450nm wavelength. A standard curve was created (www.elisa analysis.com/) and LRG1, as well as HBB concentrations were calculated. Significance was determined by one-way ANOVA. P values < 0.05 were considered statistically significant.

Logistic regression modeling was employed to construct receiver operator curves (ROC) to examine the predictive performance of LRG1 in relation to the diagnoses of TBI, PTSD and TBI+PTSD. ROC curve comparisons were based on area under the curve (AUC), SE, and the associated 95% confidence interval (CI). Subsequently sensitivity of the various diagnostics using the predicted probability of each subject by logistic regression modeling with specificity of at least eighty percent was calculated. All analyses were conducted using the SPSS version 24.0 for Macintosh.

2.3 Results

The baseline demographics for the cohort of active-duty soldiers investigated in this study are given in Table 2-1. After screening 458 soldiers, a total of 120 samples were included in the study, containing controls (n=52), subjects with a history of TBI (n=21), PTSD (n=34) and TBI+PTSD (n=13). Figure 2-4 shows the recruitment disposition chart. No difference was observed for mean age between the described groups. Education (in years) was slightly higher within the TBI+PTSD group compared to control, TBI and PTSD subjects ($p=0.035$). No significant difference was observed for ethnicity among groups. There were no significant differences between the controls and TBI, PTSD, and TBI+PTSD subjects' age and education. All participants were male. With regard to prior deployments, we ensured during our experimental design that not

only the TBI, PTSD, and TBI+PTSD cohorts comprised soldiers with previous deployments, but the control group also was composed of soldiers who had been previously deployed to the Iraq/Afghanistan conflicts (n=35 control subjects, n=14 in the TBI group, n=23 in the PTSD group, and n=13 in the TBI+PTSD group). Subjects were included if they sustained a TBI during military training, which is common among active duty soldiers or from previous deployments. Control subjects had no history of TBI and/or PTSD, depression, alcohol abuse or sleep deprivation. Chi-square analysis revealed that prior deployment status was not significantly different across the diagnostic categories (p=0.213).

	Control n=52	**TBI** n=21	**PTSD** n=34	**TBI+PTSD** n=13	**Chi square**
Age (M, SD)	26.7 ± 6.9	25.9 ± 6.3	26.4 ± 7.4	29.9 ± 5.6	*p = 0.416*
Education (M, SD)	12.9 ± 1.3	13.0 ± 1.4	12.9 ± 1.4	14.2 ± 1.7	*p = 0.051*
Male	52	21	34	13	
Ethnicity					*p = 0.903*
White (%)	39 (75.1)	15 (71.5)	25 (75.8)	10 (83.3)	
African American (%)	2 (3.8)	2 (9.5)	4 (12.1)	0 (0)	
Hispanic (%)	5 (9.6)	2 (9.5)	3 (9.1)	2 (16.7)	
Others (%)	6 (11.5)	2 (9.5)	1 (3.0)	0 (0)	

Table 2-1 Baseline demographics for clinical cohort. Recruited subjects were all male active duty soldiers. No differences in age, education and overall ethnicity were observed among the diagnostic groups.

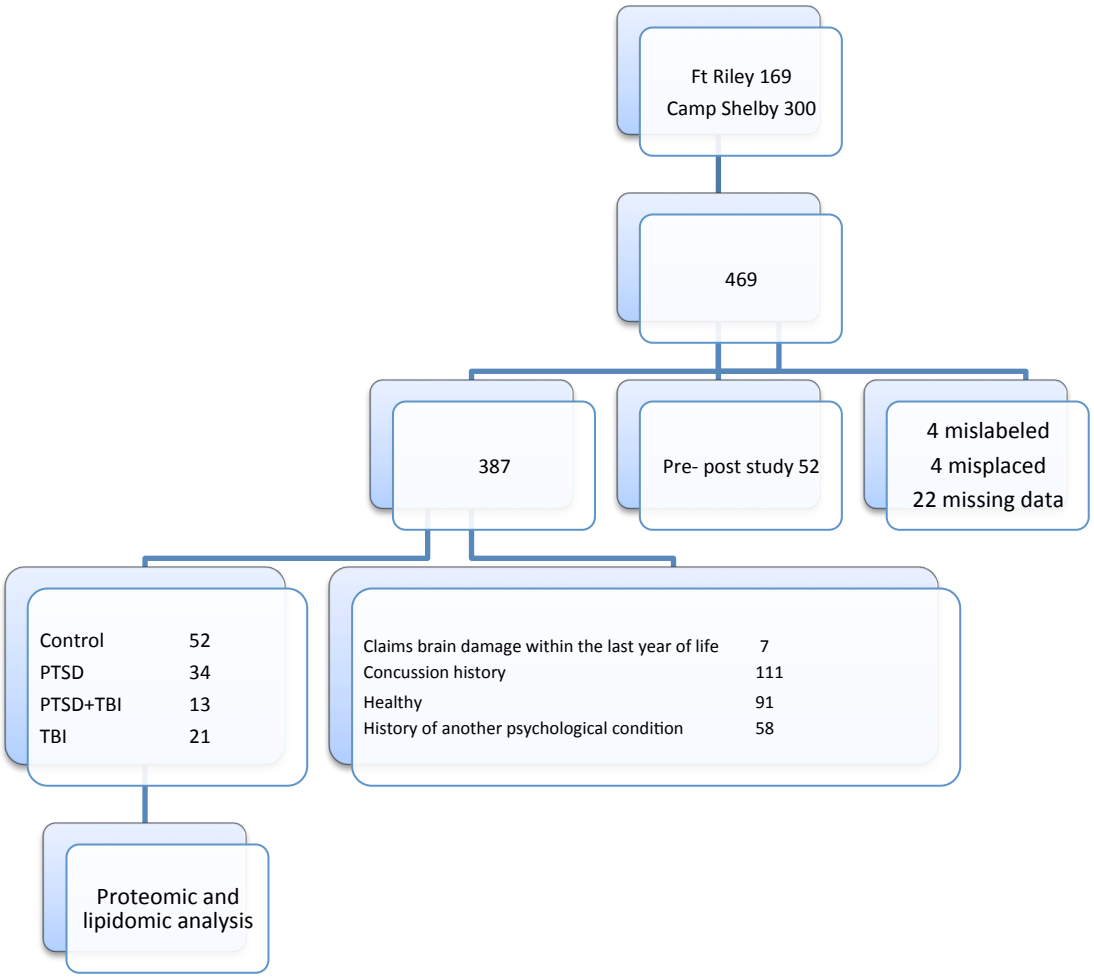


Figure 2-4 Disposition chart: A total of 469 subjects were recruited into the study. 4 Samples were misplaced, 4 mislabeled and 22 samples had missing data. 52 subjects were excluded from the study, as they are being used for another pre and post deployment study paradigm. From the 387 remaining subjects 267 subjects were excluded, as 58 subjects showed a history of other psychological conditions (e.g. anxiety, depression) or undocumented PTSD history, 111 had undocumented, not military related concussions, claimed to have brain damage within the last year but did not receive a TBI diagnosis (n=7). 91 subjects were excluded, as they are being used in other studies. The remaining 120 subjects were included in omic analyses.

Neuropsychological measurements

Category averages for the neuropsychological measurements are presented in Table 2-2. For positive PTSD diagnosis, participants had to score ≥35 on the PCL-M. PCL-M scores were significantly higher for the PTSD (45.1 ± 1.4) and TBI+PTSD (42.8 ± 1.6) groups compared to controls (19.8 ± 0.5) and TBI only (21.6 ± 1.5) ($p<0.001$ for PTSD and TBI+PTSD compared to TBI and controls). Data was normally and not biphasic distributed. Participants positive for PTSD also had significantly higher scores in anxiety ($p<0.001$), depression ($p<0.001$), sleepiness ($p<0.001$) as well as exhibited lower sleep quality ($p<0.001$). There were no differences in groups regarding alcohol consumption ($p=0.19$) and the Neurocognitive Composite Index ($p=0.98$).

Subjects within the TBI and TBI+PTSD group had sustained TBIs, which were categorized as mild TBIs resulting from one or more of the following: fragment, bullet, vehicle, fall, Improvised Explosive Device (IED), rocket-propelled grenade (RPG), land mine or grenade. Symptoms at the time of injury included: being dazed, confusion, not remembering the injury, loss of consciousness up to a maximum of thirty minutes, as well as continuous symptoms of concussion post injury (headaches, dizziness, irritability etc.) and at the current time of participant recruitment and sample collection. Of ongoing symptoms 42% of subjects who had sustained a TBI reported sleep problems and headaches, 38% experienced memory problems, 29% heard ringing in their ears, 24% showed irritability. Less common symptoms included dizziness (19%) and balance problems (14%). Control subjects scored negative on all traumatic stress questionnaires and had sustained no TBI.

	Control	TBI	PTSD	TBI+PTSD	Chi-square
PCL-M	19.8 ± 0.5	21.6 ± 1.5	45.1 ± 1.4	42.8 ± 1.6	*p < 0.001*
ZDS	31.7 ± 0.8	34.7 ± 2.4	46.5 ±1.5	41.1 ±1.5	*p < 0.001*
ZAS	29.4 ± 0.7	32.5 ± 2.1	37.1 ± 1.6	33.8 ± 2.2	*p < 0.002*
ESS	7.5 ± 0.6	9.1 ± 1.2	11.0 ± 0.8	8.4 ±1.0	*p < 0.0093*
PSQI	5.2 ± 0.3	6.9 ± 0.5	8.2 ± 1.1	7.4 ± 0.9	*p < 0.001*
AUDIT	6.0 ± 0.6	6.2 ± 1.2	8.4 ± 1.1	7.2 ±1.3	*p = 0.19*
CNS-VS	72.7 ± 4.8	72.6 ± 5.2	72.9 ± 3.9	69.4 ± 8.7	*p = 0.98*
Total previous deployments	1.0 ± 0.1	1.1 ± 0.2	1.1 ± 0.2	1.9 ± 0.2	*p = 0.213*

Table 2-2 Neuropsychological measurements in mean ± SEM. Subjects scoring positive for PTSD had significantly higher scores at the PCL-M (score≥35; $p<0.001$), as well as higher scores for ZDS ($p<0.001$), ZAS ($p<0.002$) and ESS ($p<0.0093$). TBI subjects scored below 35 at the PCL-M and scored positive for TBI on the *BTBIS*. Control subjects had PCL-M scores below 35 and no sustained TBI. No differences were observed for AUDIT and CNS-VS screening between groups.

Proteomic analysis

We performed quantitative proteomics, by using TMT isotopic labeling of trypsin digests and separation via LC, which was followed by MS^2 to quantify any global protein changes, which correlated with TBI and/or development of PTSD. A comparative analysis between controls and the three groups (1) TBI diagnosis 2) PTSD diagnosis 3) TBI/PTSD diagnosis, was performed using the workflow described in Figure 2-1. We identified a total of 703 peptides in at least eight 10-plex groupings. 136 proteins were included in the statistical analysis and 4 reverse sequence proteins and one contaminant and one human albumin were excluded. Before multiple test correction was applied three proteins reached significance (see below), however after post-hoc analysis no protein passed the threshold (Table 2-3).

Description	Accession	Prob > F	FDR Adjusted p Value
Leucine-rich alpha-2-glycoprotein	P02750	0.014	0.45
Hemoglobin subunit beta	P68871	0.024	0.97
Vitamin K-dependent Protein S	P07225	0.036	0.97

Table 2-3 Statistically significant proteins identified by ANOVA before post-hoc analysis and with corrected FDR p-value**.**

In Figure 2-5 a-c scatterplots of these 3 proteins are shown, as identified by LCMS. MS data is represented as log2 (data) of subject/channel 131. Although significance was not achieved after post-hoc analysis, we decided to perform ELISA on Leucine-rich alpha-2-glycoprotein (LRG1), as the scatterplot indicated a higher trend for the TBI+PTSD group (data represented in log2(mean concentration)).

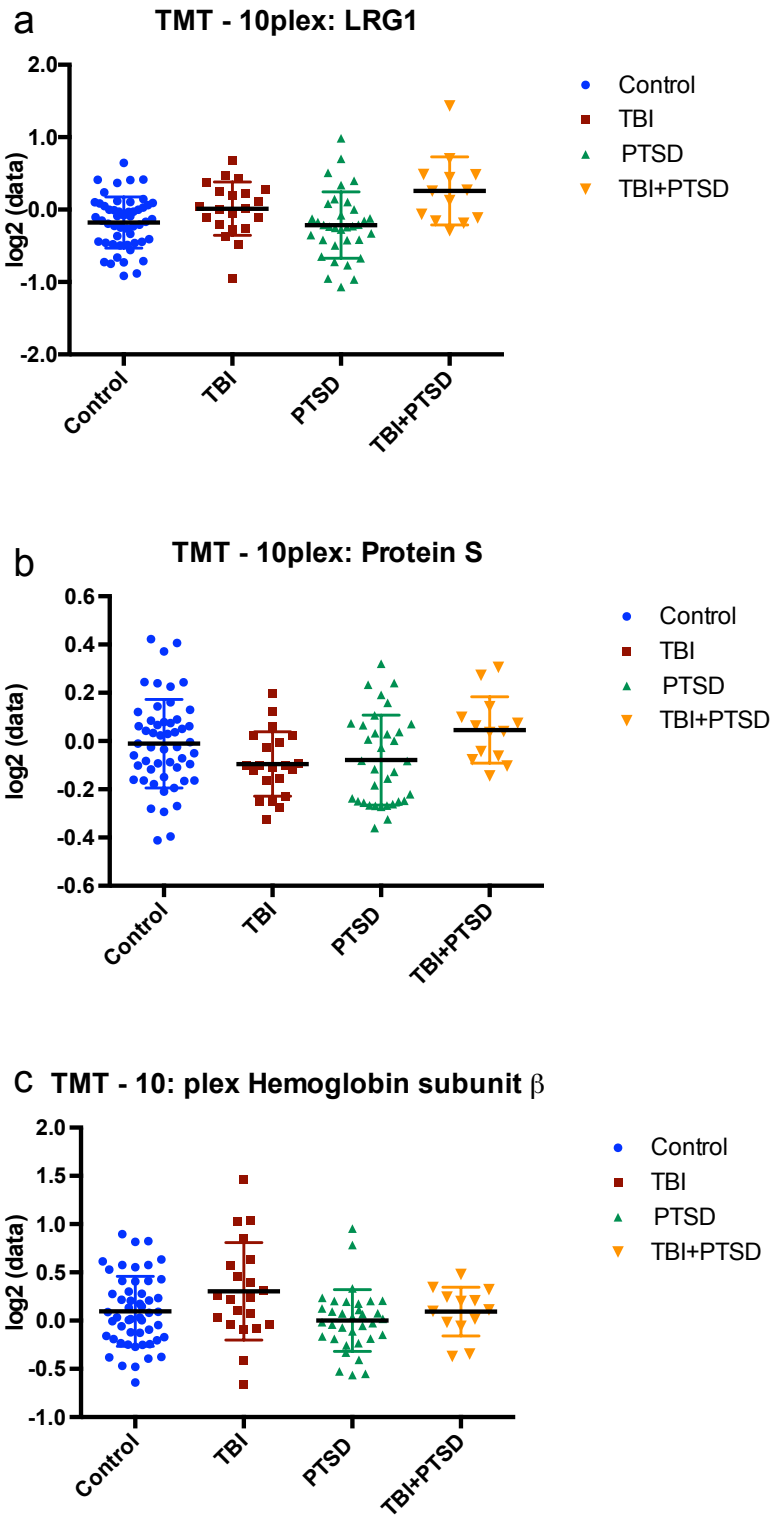


Figure 2-5 Scatterplots of proteins, identified via LCMS, which reached significance after ANOVA, before post-hoc a) LRG1 b) Vitamin K dependent Protein S c) Hemoglobin subunit β. Data are represented in log2 of (subject/channel 131) for MS analysis.

The ELISA revealed significantly elevated levels of LRG1 in TBI, PTSD and TBI+PTSD subjects compared to controls (Prob>Chi square: TBI: $p < 0.001$; PTSD $p = 0.001$; TBI+PTSD $p<0.001$; Figure 2-6). Mean (and SEM) concentration for LRG1 in plasma was observed as follows: Control: 33.7 ± 1.5 µg/ml; TBI: 57.1 ± 3.3 µg/ml; PTSD: 43.3 ± 2.1 µg/ml; TBI+PTSD: 95.1 ± 5.1 µg/ml.

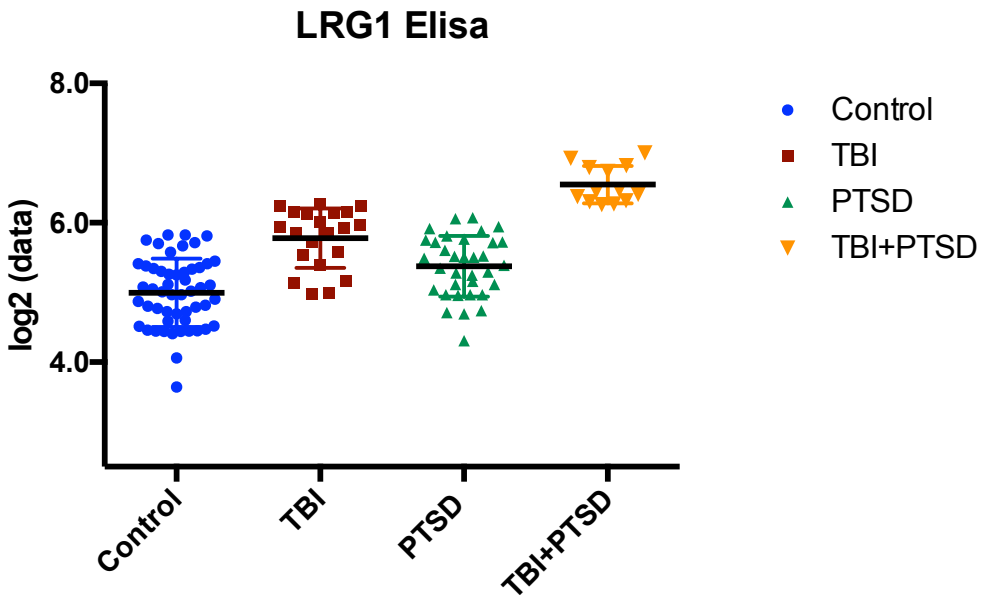


Figure 2-6 ELISA LRG1 in subjects with TBI, PTSD and TBI+PTSD. LRG1 levels were significantly elevated in TBI, PTSD and TBI+PTSD patients (data represented in log2 (mean concentration).

Examination of sensitivity and specificity using ROC analysis revealed the AUC for the different diagnostics/control as follows: For TBI/Control the AUC was 0.886 (95% CI [0.802–0.971], p< .001). For PTSD/Control the AUC was 0.715 (95% CI [0.605–0.825], p< 0.05). When investigating TBI+PTSD/Control subjects, the AUC was increased to 1.000 (95% CI [0.86–0.95], p< .001). The various ROC curves are displayed in Figure 2-7. Note: As ranges of LRG1 concentration of control and TBI+PTSD subjects did not overlap, a ROC curve could not be created at this point. Optimal sensitivities with

specificity of at least 80% predicted probabilities are shown in Table 2-4. The highest sensitivity and specificity was achieved with LRG1 for TBI+PTSD/Control. ROC tables, containing all values for sensitivity and 1-specificity can be found in the appendix section 2.

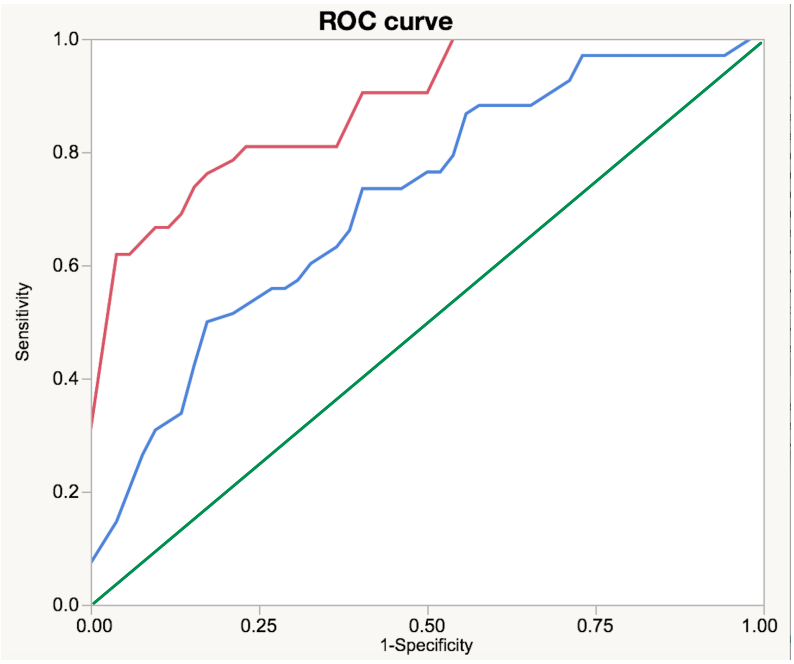


Figure 2-7 ROC curves for TBI/Control (red) and PTSD/Control (blue). TBI+PTSD/Control is not displayed.

Model (LRG1 concentration)	Sensitivity	Specificity	# R^2
TBI/Control	76%	80%	0.52
PTSD/Control	50%	80%	0.19
TBI+PTSD/Control	100%	100%	1.00

Table 2-4 Optimal sensitivities with specificities at least 80% for the various diagnostic models*.

*Calculations based on predicted probabilities form binary logistic Regression.
Represents Nagelkerke R^2

We further used the same regression analysis to develop models for PTSD/TBI, TBI+PTSD/TBI and PTSD/TBI+PTSD prediction. ROC analysis revealed the AUC for as follows: For PTSD/TBI the AUC was 0.767 (95% CI [0.633–0.900], $p < 0.05$). No model could be deployed for TBI+PTSD/TBI and TBI+PTSD/PTSD, as LRG1 concentration did not pass significance test during linear regression. The ROC curve for the PTSD/TBI model is displayed in Figure 2-8. Table 2-5 shows specificity of 80% predicted probabilities for PTSD/TBI. ROC tables can be found in the appendix section 2.

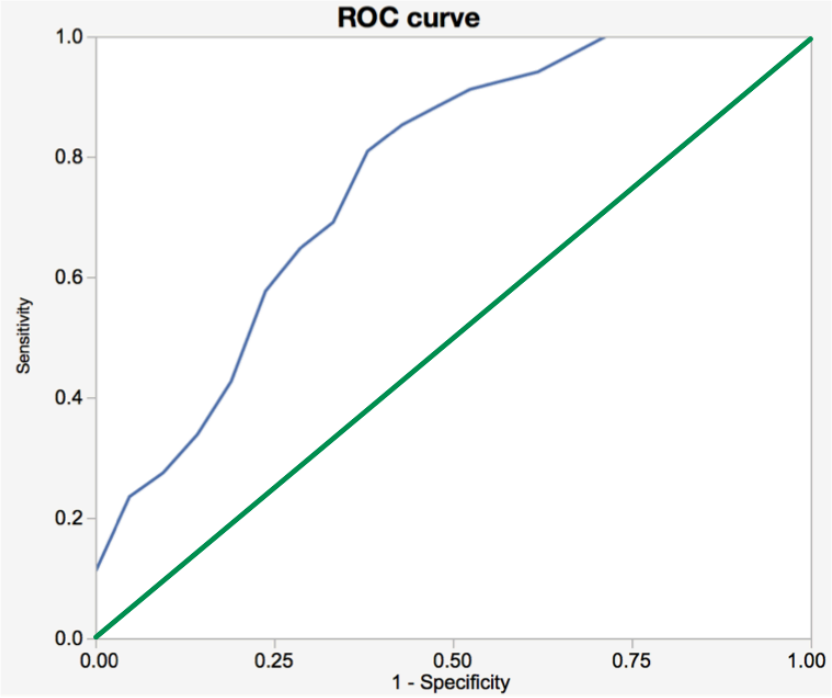


Figure 2-8 ROC curve for PTSD/TBI.

*Model	Sensitivity	Specificity	# R^2
PTSD/TBI	50%	80%	0.241
TBI+PTSD/TBI	NA	NA	NA
TBI+PTSD/PTSD	NA	NA	NA

Table 2-5 Optimal sensitivities with specificities at least 80% for the various diagnostic models*.

*Calculations based on predicted probabilities form binary logistic Regression.
Represents Nagelkerke R^2

As significance was achieved for LRG1 we also employed an ELISA for the second most significant protein on the list before multiple test correction (HBB). Using this antibody-based approach for HBB, no significant difference was observed between control, TBI, PTSD and TBI+PTSD subjects (Figure 2-9). Mean (± SEM) concentration for HBB in plasma was observed as follows: Control: 13.8 ± 0.5 ng/ml; TBI: 13.1 ± 0.6 ng/ml; PTSD: 14.0 ± 0.5 ng/ml; TBI+PTSD: 12.9 ± 0.6 ng/ml.

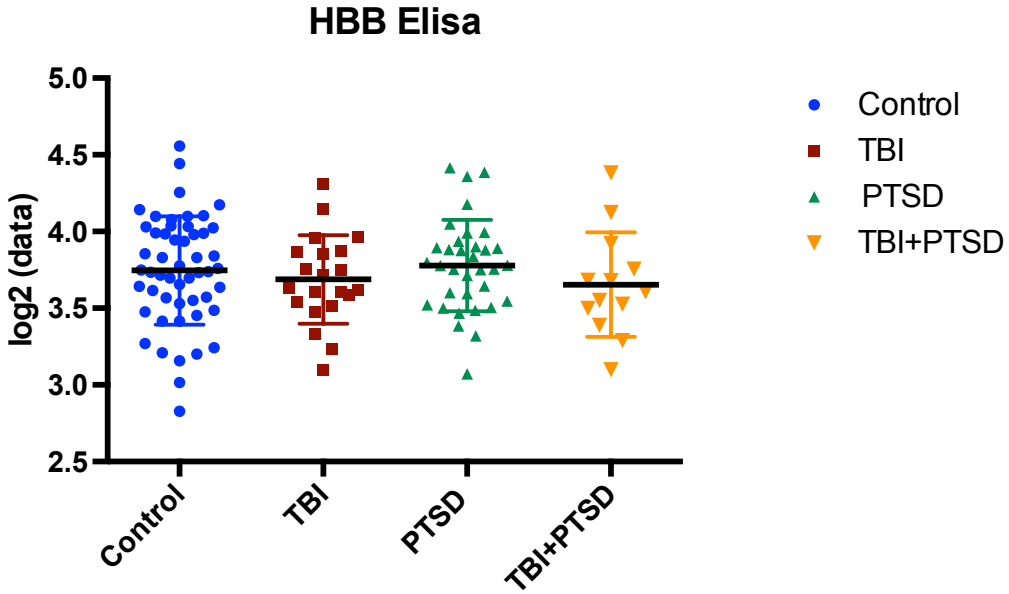


Figure 2-9 ELISA HBB in subjects with TBI, PTSD and TBI+PTSD. Plasma levels of HBB showed no differences across TBI, PTSD and TBI+PTSD patients or controls (data represented in log2(mean concentration).

As HBB, being the second most significant protein on the list before FDR correction, did not reach significance for any diagnostic group, no ELISA was performed for Protein S.

2.4 Discussion

In this study we tried to evaluate if mTBI and PTSD, and biomarkers unique to their chronic presentation, are detectable in the plasma and influence protein changes, which can be used for diagnosis or to identify potential therapeutic approaches. The field of proteomics has developed rapidly in the past decade, leading to the discovery of biomarkers not previously available [270]. The global proteomics approach used in this analysis allowed us to objectively investigate changes in control versus disease state, in order to identify distinctive protein biomarkers uniquely up- or down-regulated.

In this cross-sectional study, we investigated plasma profiles in active-duty soldiers that had a history of mild TBI, PTSD, or both, and controls (with respect to TBI and/or PTSD).

We chose plasma over serum, because plasma can be processed immediately. Serum has to clot before it can be processed and stored. The time from collection to processing and freezing leads to an increase in sample complexity due to the activity of degrading enzymes. This can result in a wrongly representation of the observed biological parameters. Furthermore, we chose a centrifugation speed of 3,200 x g RCF. Our protocols are optimized to ensure that there is no coagulation and that shearing of

cells does not occur due to high centrifugation speed, to which mammalian cells are particularly sensitive, and which could contribute to generating cellular debris [271].

Another important factor, which can potentially influence the results of our study, is the time of sample collection. Many parameters, such as peptide hormones and cytokines have diurnal rhythms. Therefore, compounds like cortisol should be measure with caution. Other analytes, such as Aβ peptides, have shown to be more stable over 24h with little intra-individual variation[272].

Three proteins (LRG1, Protein S, and Hemoglobin) were identified to be significantly different in the plasma of subjects across these diagnostic categories, however the false discovery rate (FDR) calculated for each protein was higher than the initial threshold established. Although the FDR was high for each of these proteins across the different diagnostic categories, we decided to further investigate the protein with the lowest p-value and FDR (LRG1) by performing antibody-based analysis with ELISA. The reasoning behind this analysis was the fact that the most stringent statistical analysis was used for data generation and that scatterplots of the data suggested an increase of LRG1 within the TBI+PTSD group. Moreover we did not want to inadvertently overlook a true positive marker due to relatively high p value caused by lack of statistical power. Furthermore, the TMT approach is limited by reporter ion ratio compression, which partially obscures the observation of significant differences within heterogeneous samples [273]. After ELISA, we observed a significant increase for TBI, PTSD and TBI+PTSD subjects for LRG1 compared to controls. In our study the TBI+PTSD group showed the largest difference to control subjects with an increase of 2.8 fold (1.7 fold for TBI and 1.3 fold for PTSD). The physiological concentrations for LRG1 in plasma of

healthy subjects ranges from 21-50 μg/ml [274]. We measured a mean of 33.7 μg/ml in our control subjects, which lies within the normal range of LRG1. If we would use the upper end of the physiological range of LRG1 (50 μg/ml), instead of our measured mean of control subjects (33.7 μg/ml) the fold increase would diminish but still be significant with approximately 1.9 fold for TBI+PTSD, whereas the data from the pure TBI and PTSD cohorts would lose their significance. While LRG1 levels in the TBI cohort gave an acceptable 76% sensitivity at 80% specificity, in the PTSD cohort the sensitivity and specificity performance for LRG1 was poor. Thus LRG1 plasma concentration was best for predicting TBI+PTSD.

The human glycoprotein LRG1 consists of 312 amino acids with a molecular weight of 34-36 kD [275]. Although LRG1 function still remains unknown it has been implicated in various roles such as in granulocytic differentiation [276] and cell adhesion [277,278], as well as in cell migration [279]. It furthermore has been shown to be involved upstream of the TGF-βR II pathway [280,281], suggesting a role in signaling and was proposed to be important for cell survival and apoptosis [274,282]. LRG1 is usually characterized as an "acute phase" protein, which is produced by the liver in response to infection or injury [274]. Although LRG1 has not been implicated in chronic TBI and PTSD physiology itself, the protein has been studied in conjunction with stress. A recent study investigated changes in gene expression levels in response to psychogenic stress in mice [283]. Findings showed stress induced up regulation of the Lrg1 gene within the hippocampus. Besides its involvement in the stress response, LRG1 has been the focus in other disease areas. For example increased expression levels of LRG1 were detected in serum of ovarium cancer patients [284,285]. LRG1 has been further suggested as a potential

biomarker for detection of epithelial ovarian cancer (EOC) as it has been shown that LRG1 is significantly increased in EOC cases compared to healthy controls [286]. Moreover, studies have shown increased LRG1 levels in the medium and low abundance serum proteins of individuals suffering from lung and pancreatic cancers [287,288]. As such, elevation of LRG1 as a biomarker for chronic TBI+PTSD might have potential but is not unique to the condition. Furthermore, although plasma is an ideal platform for biomarker discovery, due to its easier accessibility, and relatively cheap analytical protocols, one challenge is the diversity in protein abundance, with a total of 90% of the whole plasma proteome being distributed across a dozen proteins (e.g., albumins and immunoglobulins). This makes the discovery of low-abundant TBI and PTSD biomarkers difficult, and as described, different techniques are required to deplete those abundant proteins before MS analysis. Yet differences in sample preparation can influence biomarker discovery, which could explain why the proteins we observed via MS did not reach significance after multiple test correction. Furthermore, one of the major challenges with this approach is the broad scattering of the quantitative data caused both by individual variations in protein abundances and in analytical variation [251], as such large cohorts are needed to confirm these data. Additionally, our cohort consists of male subjects only. In the future, female subjects should be included in the analysis.

Finally, LRG1 concentration should be investigated in mouse models of the described conditions. In Chapter 4 we will observe LRG1 in a mouse model of mTBI. Furthermore, animal models of PTSD, as well as TBI+PTSD are being developed at the Institute. In the future we will expand our analyses to validate LRG1 as a potential marker.

Finally, our cross-sectional study is a case vs. control study. Subjects were categorized into different diagnostic groups and compared on the bases of the described attributes (Method section). This type of observationally study allows for the identification of factors that contribute to the condition and have the advantage of being less costly with fast sample collection. Case-control studies are also used for epidemiological studies. They also can have the advantage of having greater statistical power, because cohort studies often have to wait for 'sufficient' number of disease events to accrue. Yet one has to keep in mind that case-control studies are observational and obtaining reliable information about a patient can be difficult, especially considering the self-report biases. Furthermore, they do not include symptom-clustering, severity of the condition or outcome observations over time. Therefore, cohort studies should be conducted in the future to gain more information about the observed changes in TBI and PTSD.

Complementary to the study of the proteome is the study of lipids (lipidomics) via MS. Lipid pathway dysfunction has been suggested to be part of the pathological underlying mechanism of TBI and PTSD (see next chapter for description), yet biomarker research of these conditions has mainly focused on the identification of protein and cytokine profiling. Thus, we suggest that the additional investigation of lipids in biological samples could add to the identification and value of TBI and PTSD biomarker research.

Chapter 3 Plasma Lipidomic Profiling in a Military Population of Mild Traumatic Brain Injury and Posttraumatic Stress Disorder with Apolipoprotein E ε4–Dependent Effect

3.0 Summary

This chapter continues the characterization of the military cohort of active duty-soldiers, which was described in Chapter 2. Soldiers were demographically matched and blood samples analyzed as before (N = 120) with mTBI, PTSD and mTBI + PTSD and those who were considered cognitively and psychologically normal. In this chapter we used liquid chromatography/mass spectrometry (LC/MS) analysis to investigate changes in phospholipids (PLs). Additionally, soldiers were genotyped for apolipoprotein E (APOE) ε4, as ApoE is a regulator of plasma lipids and APOE genotype has been associated with TBI outcome. We observed significantly lower levels of several major PL classes in TBI, PTSD and TBI+PTSD compared to controls. PTSD severity analysis revealed that significant PL decreases were primarily restricted to the moderate-to-severe PTSD group. An examination of the degree of unsaturation showed that monounsaturated fatty acid (MUFA) containing phosphatidylcholine (PC) and phosphatidylinositol (PI) species were lower in the TBI and TBI+PTSD groups. However these PLs were unaltered among PTSD subjects compared to controls. Similarly, ether PC (ePC) levels were lower in PTSD and TBI+PTSD subjects relative to controls. Ratios of arachidonic acid (AA) to docosahexaenoic acid (DHA) containing species were significantly decreased within PC and PE classes. APOE ε4 (+) subjects exhibited higher

PL levels than their APOE ε4 (-) counterparts within the same diagnostic groups. These findings suggest that PL profiles, together with APOE genotyping, could potentially aid differential diagnosis of mTBI and PTSD.

3.1 Introduction

Lipids are defined as organic compounds that are poorly soluble in water but miscible in organic solvents. They are a class of biomolecules that play a crucial and highly diverse role, not only structural as a major constituent of cell membranes, but also in various metabolic pathways. They are involved in regulation of membrane proteins, membrane trafficking, cellular architecture and creating specific sub-compartments in membranes that contribute to cellular function [289]. Thus, it is logical to assume that lipids play an important role in various disease and injury pathways. Although the exact mechanisms of how lipid complexity affects cell homeostasis remains unclear, the emerging field of lipidomics has gained much attention in recent years by the scientific community. Furthermore, due to their involvement in various biological processes, lipids have become an interesting treatment target and clinical studies have started to investigate the effects of dietary lipid supplementation (docosahexaenoic acid **(**DHA)) in injury and disease.

Sphingomyelin and glycerophospholipids, together defined as phospholipids (PLs), are the most abundant lipid constituents in plasma, and also represent 20-25% of the dry weight of the adult brain and 50–60% of the total membrane mass, with proteins accounting for most of the rest [290]. The structure of PLs contains a glycerol backbone (except for sphingomyelin (SM), which has a sphingosine backbone) with hydroxyl groups esterified at positions 1 and/or 2 with long-chain fatty acids (FA). The first position of the glycerol backbone (sn1) contains in general a saturated fatty acid (e.g., palmitic or stearic acid), whereas the second position of the glycerol backbone (sn2)

contains unsaturated fatty acids, such as arachidonic acid (AA) and docosahexaenoic acid (DHA) [290]. Among other things, the degree of saturation of these lipids affects membrane fluidity. For instance, an increase in saturated lipids elevates viscosity while an increase in unsaturated lipids leads to elevated membrane fluidity [290]. Phospholipids are divided into subclasses based on their unique polar head group, which is linked to the remaining position of the glycerol backbone (Figure 3-1). Thus for the phospholipids phosphatidylcholine (PC) and SM (on sphingosine backbone), the last position contains a choline moiety. For phosphatidylethanolamine (PE) an ethanolamine can be found at this spot and for phosphatidylinositol (PI) an inositol group. Therefore, PLs are amphipathic molecules, having a polar head group and non-polar FA chains.
Lysophosphatidylcholines (LPC) and lysophosphatidylethanolamines (LPE) are phospholipids derived from PC and PE respectively via phospholipases [291]. In blood, LPC and LPE are complexed to albumin and lipoproteins, comprising 5-20% of total PL. Unsaturated LPC and LPE are mainly released by the liver and their role is to supply extrahepatic tissues with both choline/ethanolamine and polyunsaturated fatty acids (PUFA) [292–294].

Phospholipids exhibit various biological functions in mammalian cells: a) Providing structural integrity through formation of lipid bilayers, b) Energy reservoir functions (e.g. triglycerides), c) Serving as precursors for second messengers such as arachidonic acid (AA), docosahexaenoic acid (DHA), ceramide, 1,2-diacylglycerol (DAG), phosphatidic acid, and lyso-phosphatidic acid [295].

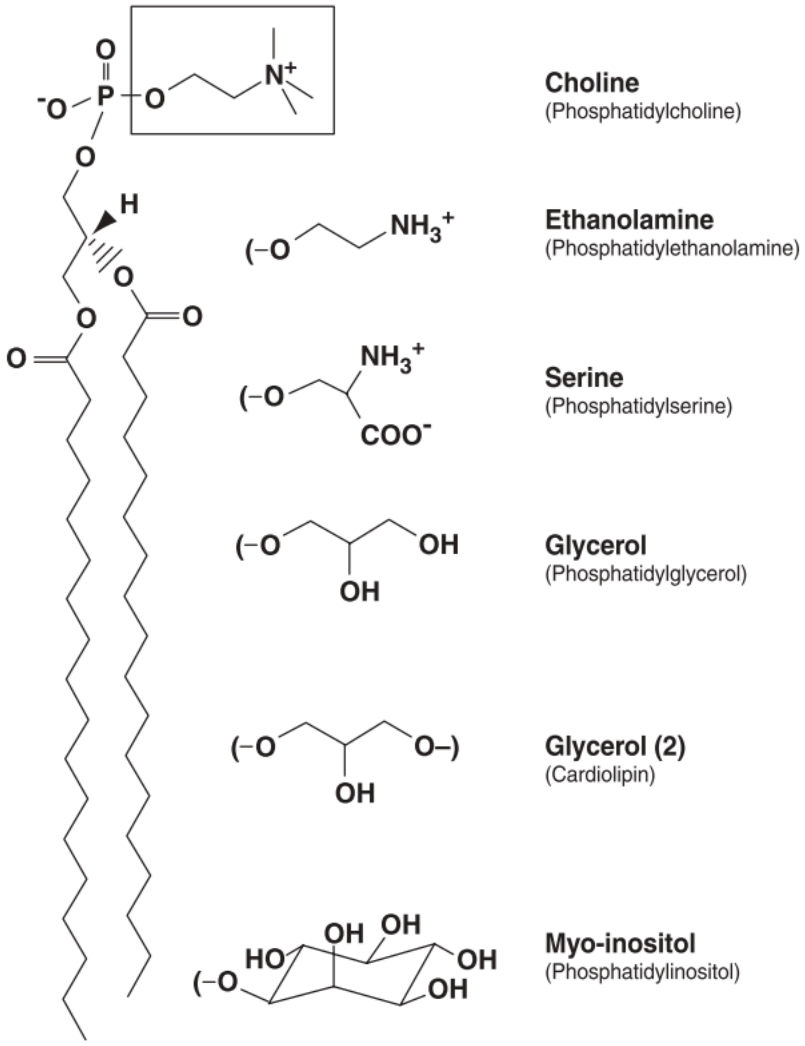


Figure 3-1 Structure of glycerophosphate-based lipids. The complete lipid structure shown is 1,2-distearoyl-sn- glycerol-3-phosphocholine or phosphatidylcholine (PC). Substitution of choline (in the box) with any of the head groups listed on the side results in other phospholipid structures as indicated. Image from [296] *Biochemistry of Lipids, Lipoproteins and Membranes.*

Ether phospholipids (ePL) have one or more of the carbon atoms on glycerol bonded to an alkyl chain via an ether linkage, as opposed to the usual ester linkage. If the ether lipid has an acetyl group instead of an acyl chain at the sn-2 position it is called platelet-activating factor (PAF). The majority of ether glycerolphospholipid species are plasmalogens [297]. Plasmalogens are glycerol-containing phospholipids with an

unsaturated *O*-(1-alkenyl) (vinyl ether) group at the sn-1 position on the glycerol chain. Plasmalogens are the most abundant form of alkenyl-glycerophospholipids, and their synthesis requires functional peroxisomes. Thus, measurement of ePLs can be seen as an indirect measure of peroxisome function.

Altered lipid metabolism is suggested to be a key event which contributes to CNS injury [298] and abnormal PL profiles have been reported in the CSF of patients with TBI. Pasvogel et al. showed that in subjects with severe TBI, levels of PC and PE were increased in the CSF of subjects who died days following injury compared to those who survived [299]. Moreover, studies on veterans with chronic PTSD showed increases in cholesterol, low-density lipoprotein, triglycerides, and a reduction of high-density lipoproteins in blood [300–303].

These studies point to a possible association between lipids and TBI and/or PTSD. Since certain lipids, such as long-chain polyunsaturated fatty acids (PUFA) are acquired from the periphery and transported to the brain, we hypothesize that a history of mTBI and/or PTSD in a military population will affect blood PL levels months after the trauma; and that lipidomic technology will be ideally suited to identify such biomarkers. Using a cross-sectional design, in this exploratory study, we performed PL profiling of plasma from active-duty soldiers with TBI and/or PTSD and healthy controls using a sophisticated lipidomic platform, which can qualitatively and quantitatively analyze several hundred PL species with high efficiency [304].

Another key regulator of plasma lipid levels is the apoE protein. More specifically, apoE is involved in the homeostatic control and distribution of plasma and tissue lipid content (Reviewed by [305]). ApoE mediates this by binding lipoproteins or

lipid complexes in plasma to specific cell-surface receptors such as the low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) receptors. The apoE isoforms (apoE2, apoE3, and apoE4) differ in their binding affinity and ability to interact with these receptors.

The APOE polymorphisms have been associated with TBI clinical outcome, which could be due to the apoE isoform specific effects with regards to lipid regulation. Multiple studies have shown that presence of the APOE ε4 allele has a negative effect on outcome following head injury and although widely accepted, these findings have not been consistently replicated (extensively reviewed by [306]) However, a recent meta-analysis of 12 studies observed a significant association of APOE ε4 with an increased risk of unfavorable long-term (<6 months) functional outcome after TBI [75]. As such it is of importance to further study APOE in conjunction with TBI in order to understand its particular role in injury.

Given the importance of the ApoE protein in lipid transport, metabolism, and the mentioned relationship to TBI, we stratified our study population to investigate any potential influence of APOE ε4 genotypes on PL profiles associated with our diagnostic categories. In this study, we explored PL profiles as a first step in the identification of mTBI and/or PTSD clinical classifications. With validation in larger cohorts, these studies could lead to the development of a biomarker panel that could be used for a differential diagnosis of mTBI and PTSD as well as comorbid cases.

3.2 Methods

Recruitment of subjects, sample collection and screening measures for the active-duty military participants is described in Chapter 2 - Method section 2.3.

APOE genotyping

The laboratory staff was blinded to any sample specific diagnostic classification during APOE genotyping. The Gentra Puregene Blood Kit (Gentra Systems) was used to purify DNA from frozen whole blood according to the manufacturer's instructions. For amplification and digestion of the APOE gene from extracted DNA, standard PCR procedure was used as described by Emi et al. [307]. The oligonucleotide primers used in this study were:

F4 (5'-ACAGAATTCGCCCCGGCCTGGTACAC-3')

F6 (5'-TAAGCTTGGCACGGCTGTCCAAGGA-3')

Each amplification reaction contained 10 pg of DNA, 1 pmol/ µl of each primer, 0.05 units/µl of AccuPrime™ GC-Rich DNA Polymerase and AccuPrime™ GC-Rich Buffer (10mM $MgSO_4$, 1mM of each dATP, dGTP, dCTP, dTTP) with water added to a final volume of 30 µl. Each reaction mixture was heated at 95°C for 5 min for denaturation, and subjected to 40 cycles of amplification by primer annealing (62°C for 1 min), extension (72°C for 2 min), and denaturation (95°C for 1 min). Polymerase chain reaction products were cleaved with restriction endonuclease CfoI (Promega, Madison, WI, USA), at 37°C overnight. Digested fragments were separated on 3% agarose gel and APOE genotypes were identified from the characteristic electrophoresis patterns.

Lipidomic Analysis

All samples were analyzed using a randomized block design (n=20) where the experimenter was blind to the diagnostic group to which each subject was assigned. Lipids were extracted from plasma samples (n = 120) using the Folch method (Folch et al. 1957). Briefly, synthetic internal standards (di-14:0 FA containing PC and PE, 14:0 FA containing (LPE) and (LPC), d18:1/17:0 SM, and di-16:0 for PI) were added to plasma prior to lipid extraction. 50µl plasma was combined with 20 vol. of chloroform: methanol (2:1), followed by centrifugation at 20,000 x g for 10min. The supernatant was transferred to a clean vial and then combined with 0.2 vol. of 0.88% Potassium Chloride Solution (KCl) followed by centrifugation. The Folch lower phase containing phospholipids was dried immediately. Lipid extracts were re-suspended in isopropanol and separation was achieved using hydrophilic interaction chromatography (HILIC) on a 1mm x 100mm column packed with 3µm Pinnacle II silica particles (Restek Corporation, Bellefonte, PA, USA). An isocratic run was performed with 70% solvent A (100% acetonitrile [ACN]) in 30% solvent B (78% methanol, 1% formic acid, 0.6% ammonium hydroxide) for 15 min at a flow rate of 55 µl/min with the column temperature at 40°C. Mass spectrometry (MS) was performed with a Thermo LTQ-XL linear ion trap mass spectrometer equipped with a Surveyor HPLC pumping system and Micro AS autosampler (Thermo-Fisher, Waltham, MA, USA). Full scan negative ion mass spectra were acquired from m/z 200 to 2,000 with in-source collision induced dissociation (SCID), with relative energies at 15%. All spectra were obtained with a 200 msec maximum ion time and by summing of 5 microscans.

Figure 3-2 shows a representative total ion chromatogram from an LC/MS run of the internal standards for PC, LPC, PE, LPE, PI and SM molecular species and Figure 3-3 shows an ion MS spectrum of PC molecular species. In order to locate peaks for each PL class on the chromatogram the m/z values corresponding to the fragment ions from each head-group were used. Furthermore, m/z 184 (the PC fragment ion produced by SCID) in the negative ion mode was additionally used to identify the PC, SM and LPC peaks, whereas PE and LPE were located on the chromatogram using m/z 140 for PE fragment ion. Phosphatidylinositols were identified using the m/z 241 PI fragment ion.

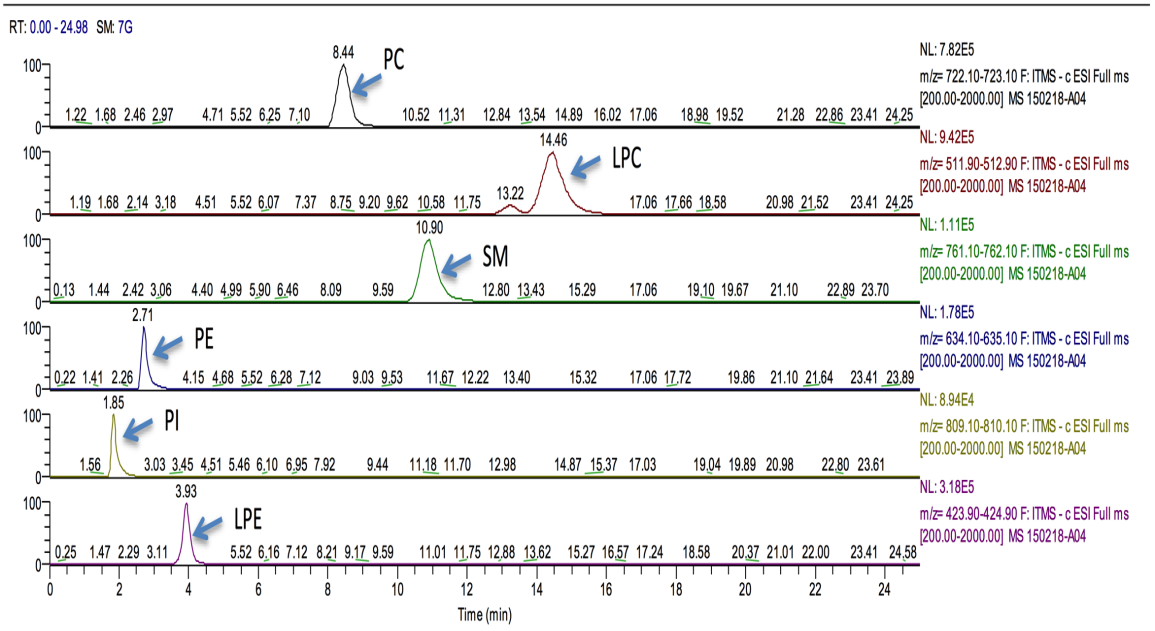


Figure 3-2 Representative total ion chromatogram: The chromatogram shows ion-plots for PC, LPC, SM, PE, LPE and PI internal standards mapping to the time where each of these PL classes elute within a 25 minute runtime. Ion plot for m/z 722.5 is $[M+CHO2]^-$ ion for PC (28:0), a di-14:0 fatty acid containing internal standard. Ion plot for m/z 512.5 is $[M+CHO2]^-$ ion for LPC, a 14:0 fatty acid containing internal standard. Ion plot for m/z 761.5 is $[M^+CHO2]^-$ ion for SM (17:0). Ion plots for PE, LPE and PI uses $[M-H]^-$ for detection with m/z 424.25 LPE (14:0), m/z 634.5 PE (28:0) and m/z 809.5 PI (a di-16:0 fatty acid containing internal standard; 32:0).

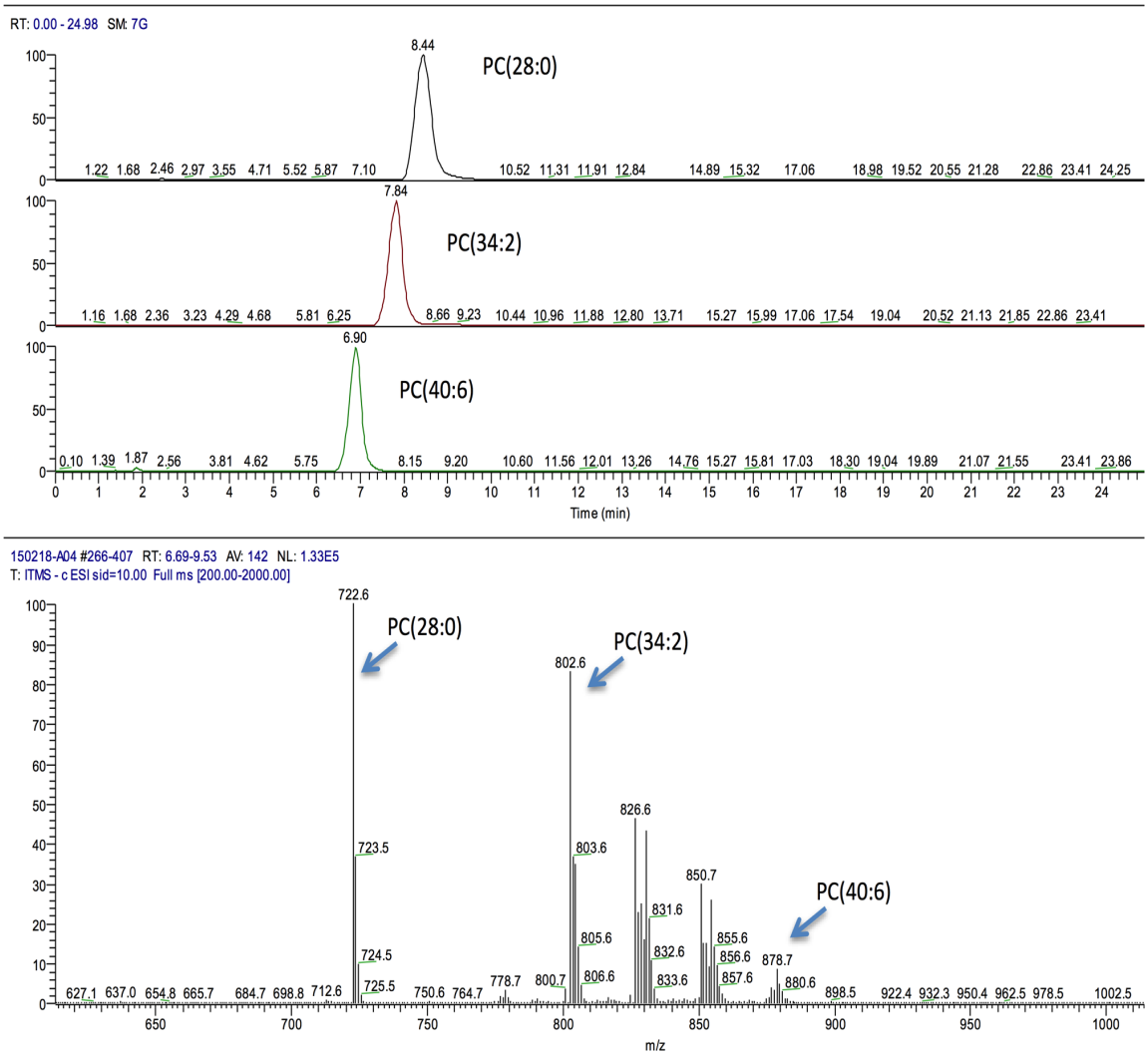


Figure 3-3 Mass Spectrometry spectra of LC/MS analysis: An image of negative ion MS spectra summed over the chromatogram covering the elution period of PC molecular species. All m/z values displayed here correspond to $[M+CHO2]^-$ ions for each PC. The m/z 722.5 corresponds to the formate adduct of PC(28:0) internal standard. MS spectra image shows PC species PC(34:2) having a m/z 802.6 and PC (40:6) having m/z 878.6. Consistent with its higher carbon number, PC(40:6) elutes earlier than PC(34:2).

Mass spectra were summed over the chromatographic peak for each PL class and spectra, (each as a list of m/z versus intensity signal) and were exported from the acquisition program to Microsoft Excel (Microsoft, Redmond, WA, USA). Files were then uploaded to LipidomeDB online (University of Kansas, Lawrence, KS, USA;

http://129.237.137.125:8080/Lipidomics/) to identify and quantify (with reference to the added internal standards without correction for response differences) each PL molecular species. The mass of target lipids and abundances of their isotopic variants were calculated from the chemical formula by adding the masses of the formate adduct ions $[M+CHO2]^-$. Located in the input data by m/z are the peaks corresponding to specified adduct ions of the internal standards and target lipids are, and the value of each corresponding signal is collected. Within a specified mass tolerance window (specified compound mass ± the mass tolerance) any located mass is considered a candidate mass for the specified target lipid or internal standard. This is followed by the search algorithm "Sum of Signals". This alorithm sums signals within the specified mass tolerance target window. Each sample was run in triplicates to control for technical variability. An independent reference sample was added to each run to control for run-to-run variability.

All molecular species identified within each PL class were summed to generate total PC, LPC, PE, LPE, SM and PI concentration values. Each phospholipid class of PC, LPC, PE, LPE, and PI was then separately grouped according to their degree of unsaturation of each molecular species (SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, and PUFA – polyunsaturated fatty acids). Sphingomyelin was excluded from this analysis due to its lack of PUFA-containing SM species.

We analyzed the ratios of arachidonic acid (AA)-containing lipid species to docosahexaenoic acid (DHA)–containing species of PC, LPC, PE, LPE and PI. For PC, we used AA-containing PC (36:4, 16:0/20:4), PC (38:4, 18:0/20:4) and PC (38:5, 18:1/20:4) to DHA-containing PC (38:6, 16:0/22:6) and PC (40:7, 18:1/22:6). For LPC, a ratio of AA-containing LPC (20:4) to DHA-containing LPC (22:6) was examined. For

PE, a ratio of AA-containing species, PE (38:4, 18:0/20:4) and ether PE (ePE 38:4, o-18:0/20:4) to DHA-containing species, PE (40:6, 18:0/22:6), and ePE (ePE; 40:6, o-18:0/22:6), was calculated. For LPE, a ratio of AA-containing LPE (20:4) to DHA-containing LPE (22:6) was used. . For (AA)-containing PI species we calculated PI (36:4, 16:0/20:4) and PI (38:4, 18:0/20:4), and for DHA-containing PI species we used PI (40:6, 18:0/22:6)/ We previously determined the identification of these species as containing AA and DHA based on MS/MS analyses [304]. Ether PC (ePC) and ePE were grouped separately, as these lipids contain ether linkage at the sn1 position.

Statistical analyses

Data were checked for normal distribution histogram plots and skewness/kurtosis measures. Statistical analyses were performed as previously described [228]. Principal Component Analysis (PCA) was used to minimize multicollinearity and achieve dimension reduction, as used routinely for the evaluation of lipidomic data. First, the Kaiser-Mayer-Olkin (KMO) and Bartlett's test for Sphericity was used to ensure sampling adequacy for PCA. Sampling adequacy as determined by a KMO value of > 0.6 and Bartlett p value < 0.05 were further investigated. Variables with eigenvalues of ≥1 were retained, PCA was used for extracting components, and varimax with Kaiser normalization was used for rotation to simplify and clarify the data structure. In order to perform mixed linear modeling (MLM) regression analysis on each component (the outcome measure), the Anderson-Rubin method was used for exporting uncorrelated scores while adjusting for random (human) factor and assess independent fixed

(diagnostic and replication). Following data analysis using MLM, Fisher's least significant difference (LSD) correction and the Benjamini–Hochberg procedure (B-H) were used for *multiple-test correction and control of false discovery rate (FDR)*. All data were analyzed using SPSS version 22.0.0 (IBM Corporation, Armonk, NY). B-H (α=0.05) was calculated using JMP 11 (SAS, Cary, NC). Data were normally distributed; therefore parametric tests were used to analyze data and presented in mean and standard derivation (SD). If data was not normally distributed, log-transformation was performed that allowed for the use of parametric testing afterwards instead of non-parametric approaches. Standard deviation was used instead of standard error of mean (SEM), as SD measures the amount of variability for a subject set of data from the mean. Meanwhile, SEM measures how far the sample mean of the data is likely to be from the true population mean, making SD the appropriate statistic to plot.

Logistic regression and ROC analysis were performed as previously described in Chapter 2 (Method section).

3.3 Results

The baseline demographic distribution of the study population can be found in chapter 2-Results section. Figure 3-4 gives the overview of lipidomic workflow of this study. Lipidomic analysis was performed on a total of 120 subjects, including controls (n = 52), and subjects with traumatic brain injury (TBI; n = 21), post-traumatic stress disorder (PTSD; n = 34) or both (n = 13). The apolipoprotein E ε4 carrier effect was investigated on all subjects. For secondary analysis, we separated TBI and PTSD

subjects. For PTSD analysis, PTSD Checklist-Military Version (PCL-M) scores were used. The data was normally and not biphasic distributed. Table 3-1 shows APOE genotype frequencies within the cohort, as well as grouped APOE ε4 (-) and APOE ε4 (+) subjects. No difference was observed for genotype frequencies (p=0.70), or for the APOE ε4 (-) and ε4 (+) distribution between controls, TBI, PTSD and TBI+PTSD subjects (p=0.84), via Chi Square and Pearson coefficient.

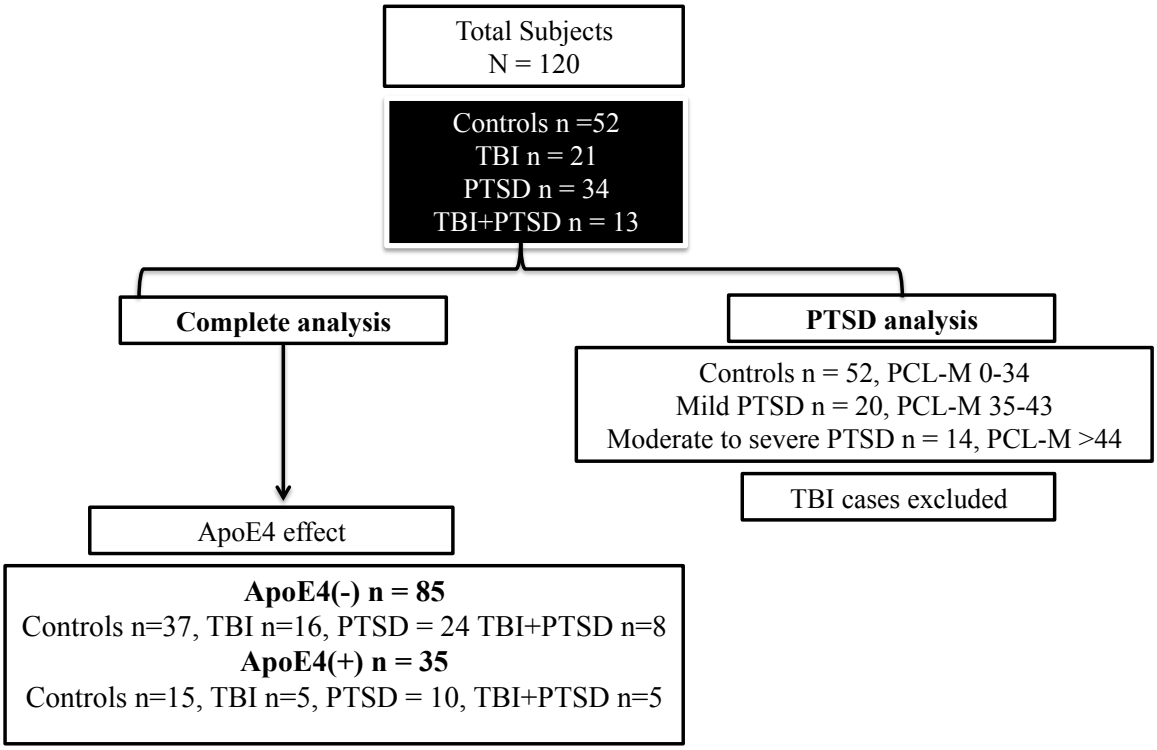


Figure 3-4 Overview and workflow of group analysis. A total of 120 subjects were recruited, including controls (n = 52), and subjects with traumatic brain injury (TBI; n = 21), post-traumatic stress disorder (PTSD; n = 34) or both TBI and PTSD (n = 13). In addition apolipoprotein E (APOE) ε4 carrier effect was investigated. For secondary analysis, we separated TBI and PTSD subjects. For PTSD analysis, PTSD Checklist-Military Version (PCL-M) scores were used.

	Control (n=52)	TBI (n=21)	PTSD (n=34)	TBI+PTSD (n=13)
APOE (n, %)				
ε2/ ε2	1 (1.9%)	0 (0%)	1 (2.9%)	0 (0%)
ε2/ ε3	3 (5.8%)	1 (4.8%)	3 (8.9%)	0 (0%)
ε2/ ε4	4 (7.7%)	0 (0%)	0 (0%)	0 (0%)
ε3/ ε3	33 (63.5%)	15 (71.4%)	20 (58.8%)	8 (61.5%)
ε3/ ε4	11 (21.1%)	4 (19.0%)	9 (26.5%)	4 (30.8%)
ε4/ ε4	0 (0%)	1 (4.8%)	1 (2.9%)	1 (7.7%)
APOE ε4 (-)	37 (71.2%)	16 (76.2%)	24 (70.6%)	8 (61.5%)
APOE ε4(+)	15 (28.8%)	5 (23.8%)	10 (29.4%)	5 (38.5%)

Table 3-1 APOE frequencies. For each diagnostic group APOE frequencies were calculated and APOE ε4 carrier (ε2/ ε4, ε3/ ε4, ε4/ ε4) and non-carrier (ε2/ ε2, ε2/ ε3, ε3/ ε3) were combined for statistical analysis.

Changes in total phospholipid content in plasma of TBI, PTSD and TBI+PTSD subjects

Comparison of plasma levels of total PC, LPC, SM, PE, LPE, and PI in subjects with TBI, PTSD, and TBI+PTSD showed significant decreases in all PL classes, compared with controls. To determine differences in total content of various PL classes for each diagnostic category (TBI, PTSD, TBI+PTSD), compared with controls, the levels of each molecular species within PC, LPC, SM, PE, LPE, and PI were summed to calculate the total for each class.

Figure 3-5 shows total levels of PC, LPC, SM, PE, LPE, and PI in the plasma subjects with TBI, PTSD, and TBI+PTSD as a percentage of control levels – SD. Overall

total PC, LPC, PE, LPE, SM, and PI were significantly decreased in TBI, PTSD, and TBI+PTSD subjects, compared with the control group.

Total PC was significantly decreased by an average of 19% in TBI subjects ($p = 0.009$), 12% in PTSD subjects ($p = 0.01$), and 24% in subjects with both TBI+PTSD ($p < 0.001$), compared with in controls. LPC was decreased by 24% in the TBI group ($p = 0.002$), 17% in the PTSD group ($p = 0.002$), and 32% in the TBI+PTSD group ($p < 0.001$). Total PE levels were decreased by 26% in TBI ($p = 0.03$), 17% in PTSD ($p = 0.01$), and 34% in TBI+PTSD ($p < 0.001$). Total LPE was decreased by 24% in the TBI group ($p < 0.001$), 17% in the PTSD group ($p = 0.001$), and 26% in the TBI+PTSD group ($p = 0.001$). Post hoc analysis showed that, relative to controls, total PI was significantly lower in the TBI group by 30% ($p = 0.008$), in the PTSD group by 19% ($p = 0.01$), and in the TBI+PTSD group by 40% ($p < 0.001$), compared with control subjects. For SM, TBI subjects showed a decrease of 17% ($p = 0.001$), PTSD subjects of 18% ($p = 0.01$), and TBI+PTSD subjects of 33% ($p < 0.001$).

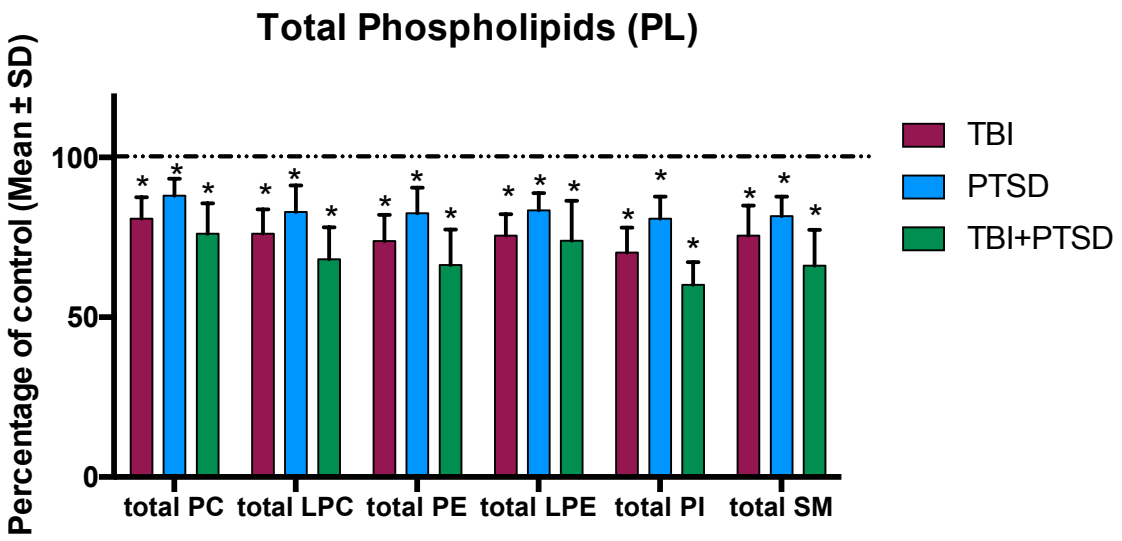


Figure 3-5 Significant changes in total plasma phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), lysophosphatidylcholines (LPC), sphingomyelin (SM), and lysophosphatidylethanolamide (LPE) in human subjects with traumatic brain injury (TBI), post-traumatic stress disorder (PTSD), or TBI and PTSD, represented by mean ± standard derivation (SD) as a percentage of control values. Individual molecular species of PC, PE, PI, LPC, SM, and LPE quantified by liquid chromatography/mass spectrometry were summed after LipidomeDB analyses to generate total lipid levels. For all species TBI, PTSD and TBI+PTSD groups were significantly decreased, compared with controls, based on mixed linear modeling regression (*denotes to $p < 0.01$).

Degree of unsaturation of PC, LPC, PE, LPE, and PI between diagnostic categories, compared with controls

We examined whether there was an effect of injury and/or PTSD on the degree of unsaturation within different PL classes (Figure 3-6). Compared with controls, we found that the degree of unsaturation of PC, LPC, PE, LPE, and PI varied significantly across different diagnostic groups. For SFA- containing PC species TBI subjects showed a decrease of 14% ($p = 0.03$), PTSD subjects of 9% ($p = 0.01$) and TBI+PTSD subjects of 17% ($p = 0.04$), compared with controls. For PUFA-containing PC species, post hoc analysis revealed a significant decrease of 17% of PUFA-containing PC species in TBI

subjects ($p = 0.03$), 10% in PTSD subjects ($p = 0.05$), and 23% in TBI+PTSD subjects ($p = 0.001$). However, MUFA-containing PC species only differed in two groups. MUFA-containing PC showed differences for TBI versus controls ($p = 0.03$) and TBI+PTSD versus controls ($p = 0.001$), differences did not reach significance for PTSD versus controls ($p = 0.06$; Fig. 3-6A).

For SFA-containing LPC species levels were decreased by 22% in TBI subjects ($p = 0.003$), 18% in PTSD subjects ($p = 0.001$), and by 34% in TBI+PTSD subjects ($p < 0.001$). For MUFA-containing LPC species, we found as well a decrease of 23% in TBI subjects ($p = 0.02$), 15% in PTSD subjects ($p = 0.02$) and 32% in TBI+PTSD subjects ($p < 0.001$). PUFA - containing LPC species were decreased by 27% in TBI subjects ($p = 0.001$), 16% in PTSD subjects ($p = 0.005$), and 28% in TBI+PTSD subjects ($p < 0.001$; Fig. 3-6B).

For SFA-containing PE, TBI subjects showed a decrease of 24% ($p = 0.02$), PTSD subjects of 19% ($p = 0.02$), and TBI+PTSD subjects of 26% ($p = 0.03$), compared with controls. For MUFA-containing PE species, post hoc revealed a significant decrease of 20% of MUFA-containing PE species in TBI subjects ($p = 0.006$), 13% in PTSD subjects ($p = 0.05$), and 23% in TBI+PTSD subjects ($p < 0.001$). For PUFA-containing PE species, we observed a significant decrease of 25% of PUFA-containing PE species in TBI subjects ($p = 0.005$), 17% in PTSD subjects ($p = 0.02$), and 35% in TBI+PTSD subjects ($p < 0.001$; Fig. 3-6C).

For SFA-containing LPE, TBI subjects showed a decrease of 18% ($p = 0.05$), PTSD subjects of 17% ($p = 0.01$), and TBI+PTSD of subjects 24% ($p = 0.06$), compared with controls. For MUFA-containing LPE, post hoc analysis revealed a significant

decrease of 23% of MUFA-containing LPE species in TBI subjects ($p < 0.001$), 12% in PTSD subjects ($p = 0.004$), and 22% in TBI+PTSD subjects ($p = 0.002$). For PUFA-containing LPE, TBI subjects showed a decrease of 27% ($p < 0.001$), PTSD subjects of 18% ($p = 0.001$), and TBI+PTSD subjects of 29% ($p < 0.001$), compared with controls (Fig. 3-6D).

For PUFA-containing PI species, TBI subjects showed a decrease of 28% ($p = 0.02$), PTSD subjects of 20% ($p = 0.01$) and TBI+PTSD subjects of 39% ($p < 0.001$). However, MUFA-containing PI species were only significantly different for TBI and TBI+PTSD, whereby post hoc comparison revealed these differences to be a decrease of 35% in TBI subjects ($p = 0.02$) and 42% in TBI+PTSD subjects ($p < 0.001$). For PTSD no significance was observed for MUFA-containing PI (Fig. 3-6E).

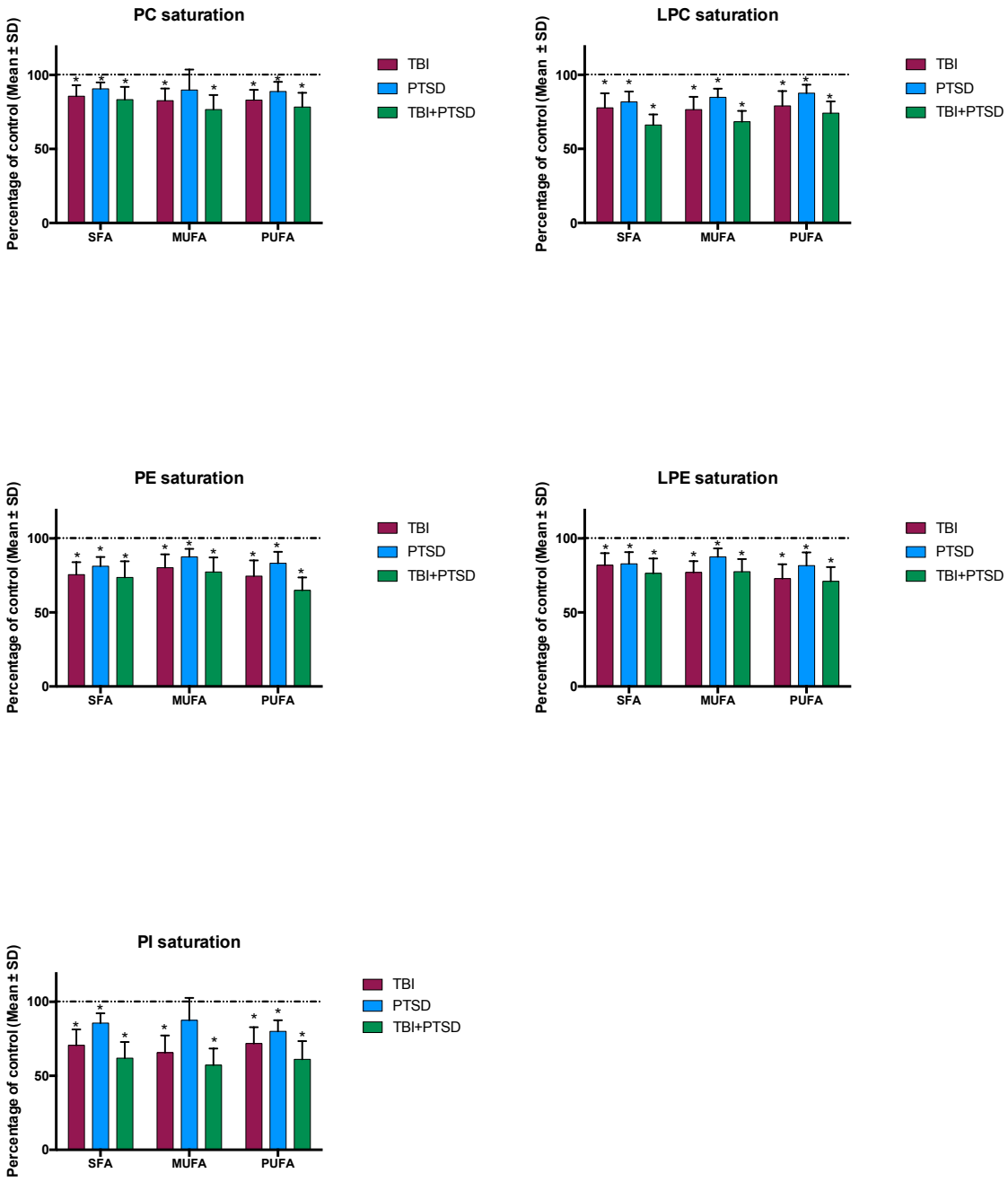


Figure 3-6 Degree of unsaturation of phospholipids (PL) classes in plasma of traumatic brain injury (TBI), post-traumatic stress disorder (PTSD), and TBI+PTSD, compared with controls. Mean ± standard derivation (SD) as a percentage of controls. (B-D) show that within the TBI, PTSD, and TBI+PTSD groups, saturated fatty acid (SFA)–containing, monounsaturated fatty acid (MUFA)–containing, and polyunsaturated fatty acid (PUFA)–containing lysophosphatidylcholines (LPC), phosphatidylethanolamine (PE), and lysophosphatidylethanolamide (LPE) were significantly decreased relative to controls. (A) and (E) show a decrease in SFA- and PUFA containing LPC and phosphatidylinositol (PI) for TBI, PTSD, and TBI+PTSD.

However, MUFA-containing LPC and PI species show only significance for TBI and TBI+PTSD. *p < 0.05; mixed linear modeling regression with post hoc analyses

As APOE genotype is known to influence plasma lipid profiles, and the ε4 allele confers risk for poor outcome after TBI, we examined the impact of an APOE ε4+ or ε4- genotype on the relationship between degree of unsaturation status of specific PL classes and the diagnostic categories TBI, PTSD, and TBI+PTSD. As such, we grouped subjects according to their APOE ε4status (APOE ε4 [-] = genotypes ε2/ ε2, ε2/ ε3, or ε3/ ε3) vs. APOE ε4 [+] = subjects with genotypes ε2/ ε4, ε3/ ε4, or ε4/ ε4; Figure 3-7).

For LPC, examination of APOE ε4genotype carrier status on SFA-, MUFA-, and PUFA-containing LPC species revealed significant differences only in MUFA-containing LPC species ($p < 0.05$). APOE ε4 (-) subjects showed significantly lower levels by 32% in TBI subjects ($p < 0.001$), 20% in PTSD subjects ($p < 0.001$), and 35% in TBI+PTSD subjects ($p < 0.001$), compared with controls, whereas levels of MUFA-containing LPC species in APOE ε4 (+) subjects showed no significant difference for the TBI and PTSD groups, compared with controls ($p > 0.05$). Only APOE ε4 (+) TBI+PTSD subjects had significantly lower levels of MUFA-containing LPC species, compared with controls ($p = 0.02$). No APOE ε4 effect was found for SFA-containing and PUFA-containing LPC species Figure 3-7A shows LPC's SFA, MUFA, and PUFA levels as a percentage of control levels for each diagnostic category in APOE ε4 carriers, that is, APOE ε4 (+) versus non-APOE ε4 carriers (APOE ε4 [-]).

For PE, APOE ε4 genotype carrier status revealed a strong effect on saturation levels of the plasma of TBI, PTSD and TBI+PTSD subjects (Figure 3-7B). After post hoc analysis, SFA-containing PE species in APOE ε4 (-) subjects showed significantly lower levels by 33% in TBI subjects ($p < 0.001$), 25% in PTSD subjects ($p < 0.001$), and 22%

in TBI+PTSD subjects ($p = 0.01$), compared with controls, whereas levels of SFA-containing LPC species in APOE ε4 (+) subjects showed no significant difference for the any diagnostic group, compared with controls ($p > 0.05$). For MUFA-containing PE species, APOE ε4 (-) subjects showed significantly lower levels by 25% in TBI subjects ($p < 0.001$), 17% in PTSD subjects ($p < 0.001$) and 19% in TBI+PTSD subjects ($p = 0.01$), compared with controls, whereas levels of MUFA-containing PE species in APOE ε4 (+) subjects showed no significant differences by diagnosis. TBI+PTSD groups remained significantly decreased, compared with controls ($p = 0.01$). For PUFA-containing PE species, APOE ε4 (-) subjects showed significantly lower levels by 32% in TBI subjects ($p < 0.001$), 20% in PTSD subjects ($p < 0.001$), and 33% in TBI+PTSD subjects ($p < 0.001$), compared with controls, whereas levels of MUFA-containing PE species in APOE ε4 (+) subjects showed no significant difference for TBI or PTSD diagnostic categories; levels of MUFA-containing PE were significantly decreased in the TBI+PTSD group compared with controls to ($p = 0.01$).

For LPE, no changes were observed for SFA-containing species (Figure 3-7C). For MUFA-containing LPE species, APOE ε4 (-) subjects showed significantly lower levels by 24% in TBI subjects ($p < 0.001$), 13% in PTSD subjects ($p = 0.006$), and 30% in TBI+PTSD subjects ($p < 0.001$), compared with controls, whereas levels of MUFA-containing LPE species in APOE ε4 (+) subjects showed no significant difference for PTSD and TBI+PTSD subjects, compared with controls ($p > 0.05$). However, the APOE ε4 (+) TBI subject group showed a significant decrease of 19% ($p = 0.03$). For PUFA-containing LPE species, APOE ε4 (-) subjects showed significantly lower levels by 25% in TBI subjects ($p < 0.001$), 19% in PTSD subjects ($p = 0.002$), and 35% in TBI+PTSD

subjects ($p < 0.001$), compared with controls, whereas levels of PUFA-containing LPE species in APOE ε4 (+) subjects showed no significant difference for TBI+PTSD, compared with controls ($p > 0.05$). However, like the APOE ε4 (-) subjects, the APOE ε4 (+) TBI and PTSD subjects, showed a significant decrease of 31% ($p = 0.03$) and 17% ($p = 0.04$) respectively.

For PC and PI, examination of APOE ε4 genotype carrier status on SFA, MUFA-, and PUFA-containing PC and PI species revealed no significant differences in TBI, PTSD, and TBI+PTSD subjects compared to controls ($p > 0.05$; data not shown).

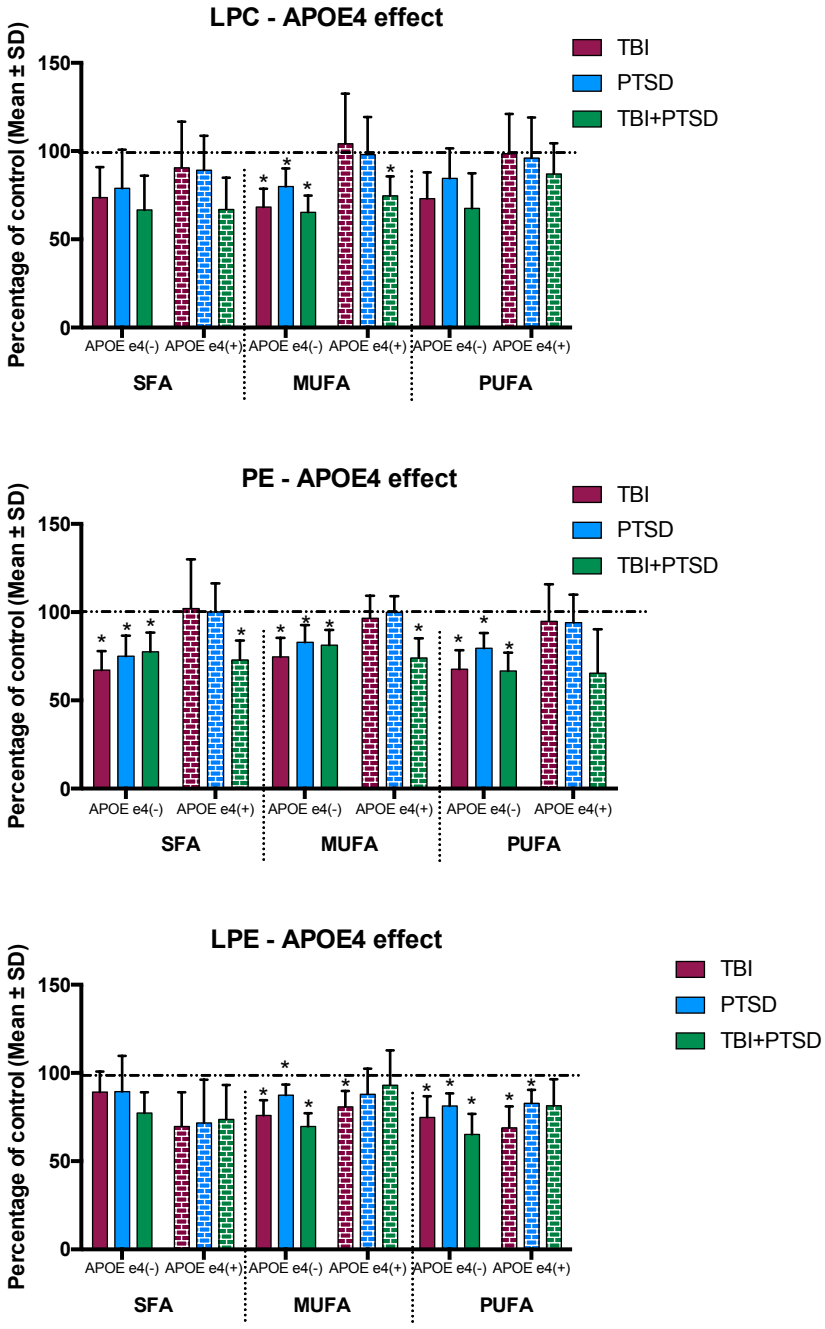


Figure 3-7Apolipoprotein E (APOE) ε4 effect on phospholipids (PL) levels in subjects with traumatic brain injury (TBI), post-traumatic stress disorder (PTSD), and TBI+PTSD, compared with controls. Mean ± standard derivation (SD) expressed as a percentage of controls. (A-C) show that within the TBI, PTSD, and TBI+PTSD groups, SFA-, MUFA-, and PUFA-containing lysophosphatidylcholines (LPC), lysophosphatidylethanolamide (LPE), and phosphatidylethanolamine (PE) are affected by APOE ε4 genotype. *p < 0.05; mixed linear modeling regression with post hoc analyses.

Examination of ePC and ePE in plasma between TBI, PTSD, and TBI+PTSD subjects, compared with controls

In order to obtain an indirect measure of peroxisome function, we examined ePC and ePE lipids. For ePC (Figure 3-8), subjects exhibiting PTSD showed a decrease of 12%, compared with controls ($p = 0.007$), whereas subjects with TBI+PTSD had a decrease of 22% ($p < 0.001$). No difference was observed for TBI ($p = 0.08$). For total ePE levels, the TBI group showed a decrease of 25% ($p = 0.01$), PTSD of 16% ($p = 0.04$), and TBI+PTSD of 35% ($p < 0.001$).

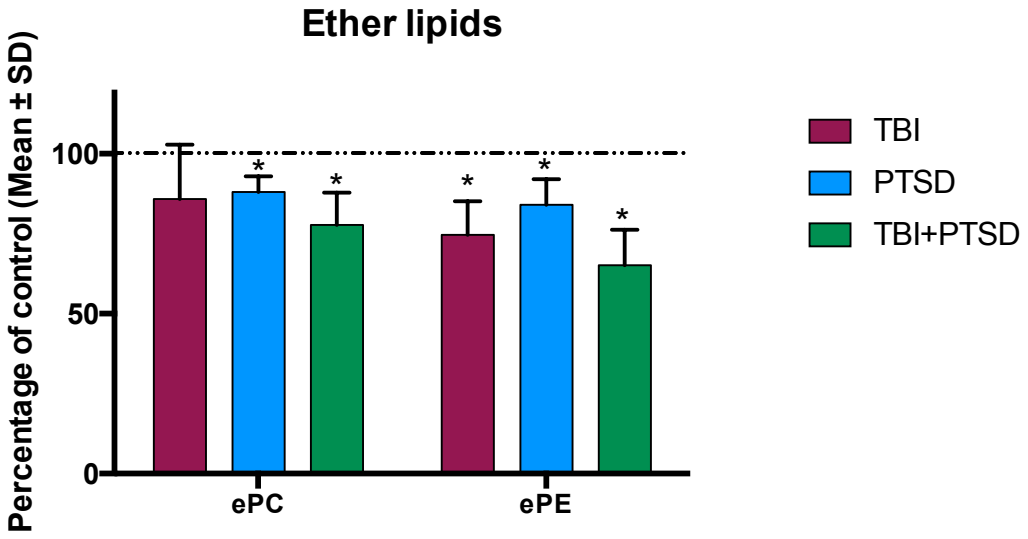


Figure 3-8 Differences in total ether phosphatidylcholine (ePC) and ether phosphatidylethanolamine (ePE) in plasma of subjects exhibiting traumatic brain injury (TBI), post-traumatic stress disorder (PTSD), or TBI+PTSD. Mean ± standard derivation (SD) as a percentage of controls. Levels of ePC were significantly decreased in the PTSD and TBI+PTSD groups, relative to controls. Levels of ePE were significantly lower in all diagnostic categories (*$p < 0.05$; mixed linear modeling regression with post hoc analysis).

In Figure 3-9, we also examined the effect of APOE ε4 genotype carrier status on ether PLs. For ePC, APOE ε4 (-) subjects showed significant lower levels by 20% in TBI subjects ($p < 0.001$), 14% in PTSD subjects ($p = 0.001$), and 25% in TBI+PTSD subjects ($p < 0.001$), compared with controls, whereas ePC levels in APOE ε4 (+) subjects of all diagnostic categories showed no significant difference, compared with controls ($p > 0.05$). The same tendency was observed for ePE, where APOE ε4 (-) subjects showed significant decreases of 34% in TBI subjects ($p < 0.001$), 20% in PTSD subjects ($p = 0.001$), and 33% in TBI+PTSD subjects ($p = 0.001$), while in APOE ε4 (+) subjects the ePC levels were only significantly reduced for TBI+PTSD ($p < 0.001$).

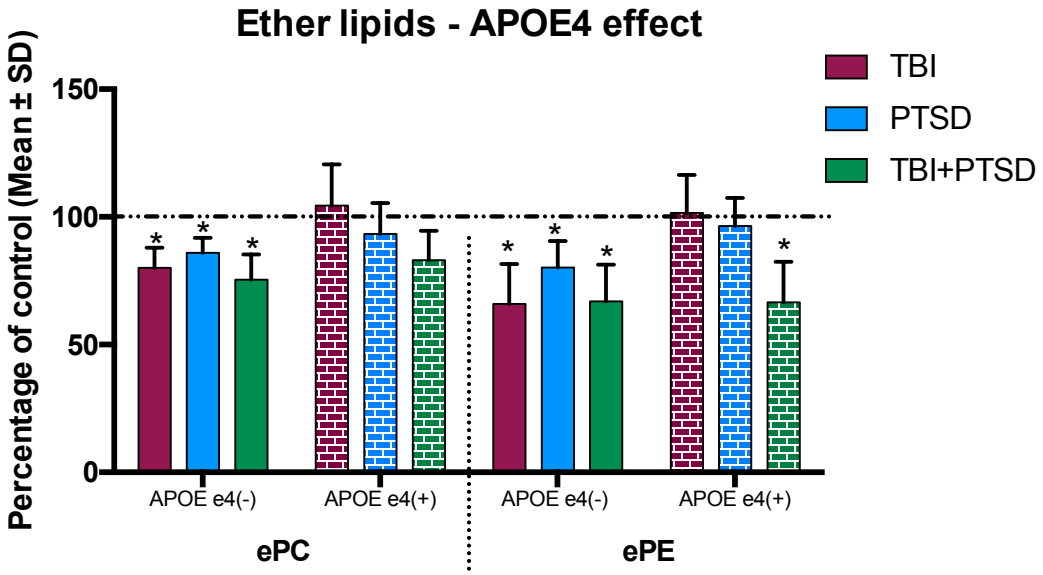


Figure 3-9 Apolipoprotein E (APOE) ε4 effect on ether lipid levels in subjects with traumatic brain injury (TBI), post-traumatic stress disorder (PTSD), and TBI+PTSD, compared with controls. Mean ± standard derivation (SD) expressed as a percentage of controls. Within the TBI, PTSD, and TBI+PTSD groups, APOE ε4 non-carriers showed significant decreases in all categories for ether phosphatidylcholine (ePC) and ether phosphatidylethanolamine (ePE) species. APOE ε4 carriers only showed significance differences in TBI+PTSD for ePC *$p < 0.05$; mixed linear modeling regression with post hoc analyses.

Ratios of AA- to DHA-containing LPC, PC, LPE, PE, and PI species between the TBI, PTSD, and TBI+PTSD groups, compared with controls

We continued our analysis by determining the ratios of AA to DHA-containing PLs and observed changes in ratios of PC and PE in plasma for TBI, PTSD, and TBI+PTSD, compared with controls (Figure 3-10), as well as the influence of APOE ε4 (Figure 3-11).

For PC, the AA to DHA ratio was significantly decreased in subjects with TBI+PTSD ($p < 0.05$). Further post hoc analysis of APOE ε4carrier status in TBI+PTSD subjects revealed the influence of APOE genotype on this finding, as the APOE ε4 (+) group showed no significant difference in the AA to DHA compared to controls ($p > 0.05$) whereas the APOE ε4 (-) subjects exhibited an AA to DHA ratio that was significantly lower than APOE genotype stratified controls by 17% ($p = 0.002$).
For LPC, there was no significant difference in the AA to DHA ratio in any of the three diagnostic categories, compared with controls ($p > 0.05$). However, further analysis revealed significant differences in the ratio in the diagnostic categories with regard to their APOE ε4carrier status. APOE ε4 (-) subjects exhibited a significantly lower AA to DHA ratio by 14% in TBI+PTSD subjects ($p = 0.002$), whereas there was no significance in APOE ε4 (-) TBI alone or PTSD alone subjects, compared with APOE genotype stratified controls. However, for APOE ε4 (+) subjects, there was a significant increase of 12% in TBI subjects ($p = 0.04$), 10% in PTSD subjects ($p = 0.04$), and 1% in TBI+PTSD subjects ($p = 0.006$), compared with controls. TBI+PTSD reached significance due to the 15% change between APOE ε4 (-) and APOE ε4 (+) subjects, compared with controls.

For PE, the AA to DHA ratio levels were lower in subjects with TBI ($p = 0.04$) and with TBI+PTSD ($p < 0.001$). Further post hoc analysis of APOE ε4 carrier status revealed no significant difference in the AA to DHA ratio for any diagnostic categories regardless of APOE ε4 carrier status.

For LPE, there was no significant difference in the AA to DHA ratio in any of the three diagnostic categories, compared with controls ($p > 0.05$), and post hoc analysis of APOE ε4 carrier status found no significant difference in AA to DHA ratio for any diagnostic categories.

For PI, there was also no significant difference in the AA to DHA ratio in any of the three diagnostic categories, compared with controls ($p > 0.05$). However, APOE ε4 (-) subjects exhibited significantly lower AA to DHA ratio by 8% in TBI subjects ($p = 0.02$), 7% in PTSD subjects ($p = 0.03$) and 16% TBI+PTSD subjects ($p = 0.01$), whereas there was no significance in APOE ε4 (+) TBI, PTSD, or TBI+PTSD subjects, compared with controls ($p > 0.05$).

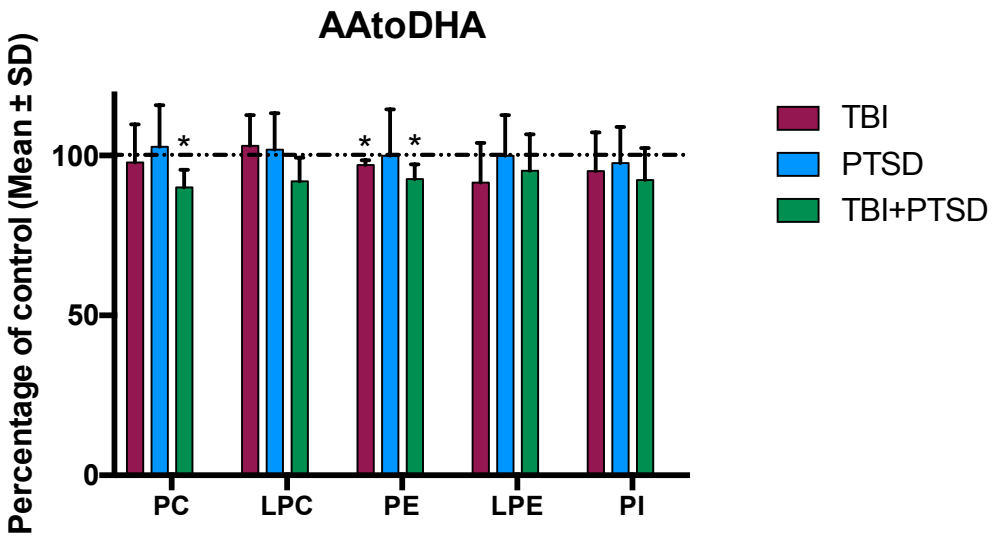


Figure 3-10 Differences in the ratios of arachidonic acid (AA)–containing to docosahexaenoic acid (DHA)–containing phospholipids (PL) in plasma of subjects exhibiting traumatic brain injury (TBI), post-traumatic stress disorder (PTSD), or TBI+PTSD, relative to control subjects. Mean ± standard derivation (SD) as a percentage of controls. Within phosphatidylcholine (PC), ratios of AA to DHA were decreased in TBI+PTSD subjects. In addition, within phosphatidylethanolamine (PE), ratios were decreased in TBI and TBI+PTSD subjects (*$p < 0.05$; mixed linear modeling regression with post hoc analysis).

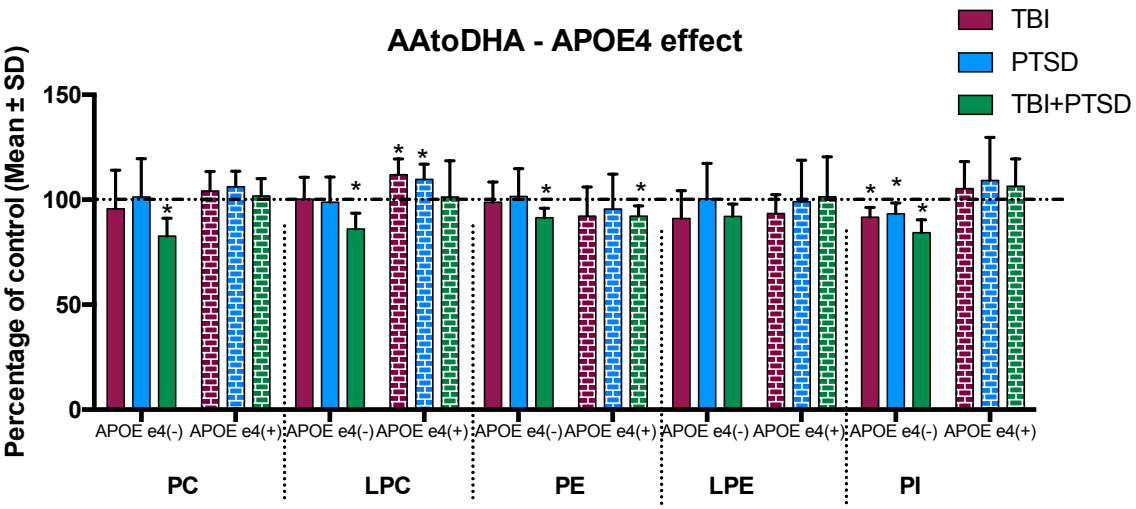


Figure 3-11 Effect of Apolipoprotein E (APOE) ε4 on ratios of arachidonic acid (AA)–containing to docosahexaenoic acid (DHA)–containing phospholipids (PL) in plasma of subjects exhibiting traumatic brain injury (TBI), post-traumatic stress disorder (PTSD), and TBI+PTSD. Mean ± standard derivation (SD) as a percentage of controls. Figure shows an APOE ε4 dependent effect on lipid levels. *$p < 0.05$; mixed linear modeling regression with post hoc analyses.

The Appendix 1 contains tables with concentrations of significant individual molecular species, which we identified in all 6 phospholipid classes (Appendix Chapter 3; Section 1: Tables 1-6). We performed a preliminary logistic regression analysis with significant species for each class to identify a model (containing multiple species) that allowed for examination of sensitivity and specificity using ROC analysis for TBI/Control, PTSD/Control and TBI+PTSD/Control. The model identified certain species for each diagnostic criterion (Table 3-2.). ROC analysis revealed the AUC for the different diagnostics as follows: For TBI the AUC was 0.892 (95% CI [0.819–0.965], $p< 0.05$). For PTSD the AUC was 0.697 (95% CI [0.587–0.807], $p< 0.05$). When investigating TBI+PTSD subjects, the AUC was increased to 0.862 (95% CI [0.754–0.970], $p< 0.05$). The ROC curves for each model are displayed in Figure 3-12.

Optimal sensitivities with specificity of at least 80% predicted probabilities are shown in Table 3-2 as well. The highest sensitivity and specificity was achieved with the model for TBI. ROC tables can be found in the Appendix, Chapter 3, Section 2.

	*Model	Sensitivity	Specifity	# R^2
TBI/Control	LPC(20:3) DSM(16:0) SM(22:0) SM(24:1)	81%	80%	0.539
PTSD/Control	ePC(40:6) LPC(16:0)	51%	80%	0.156
TBI+PTSD/Control	PC(36:5) ePE(34:0)	81%	80%	0.404

Table 3-2 Optimal sensitivities with specificities of at least 80% for the various diagnostic models*.

*Calculations based on predicted probabilities form binary logistic Regression.
Represents Nagelkerke R^2

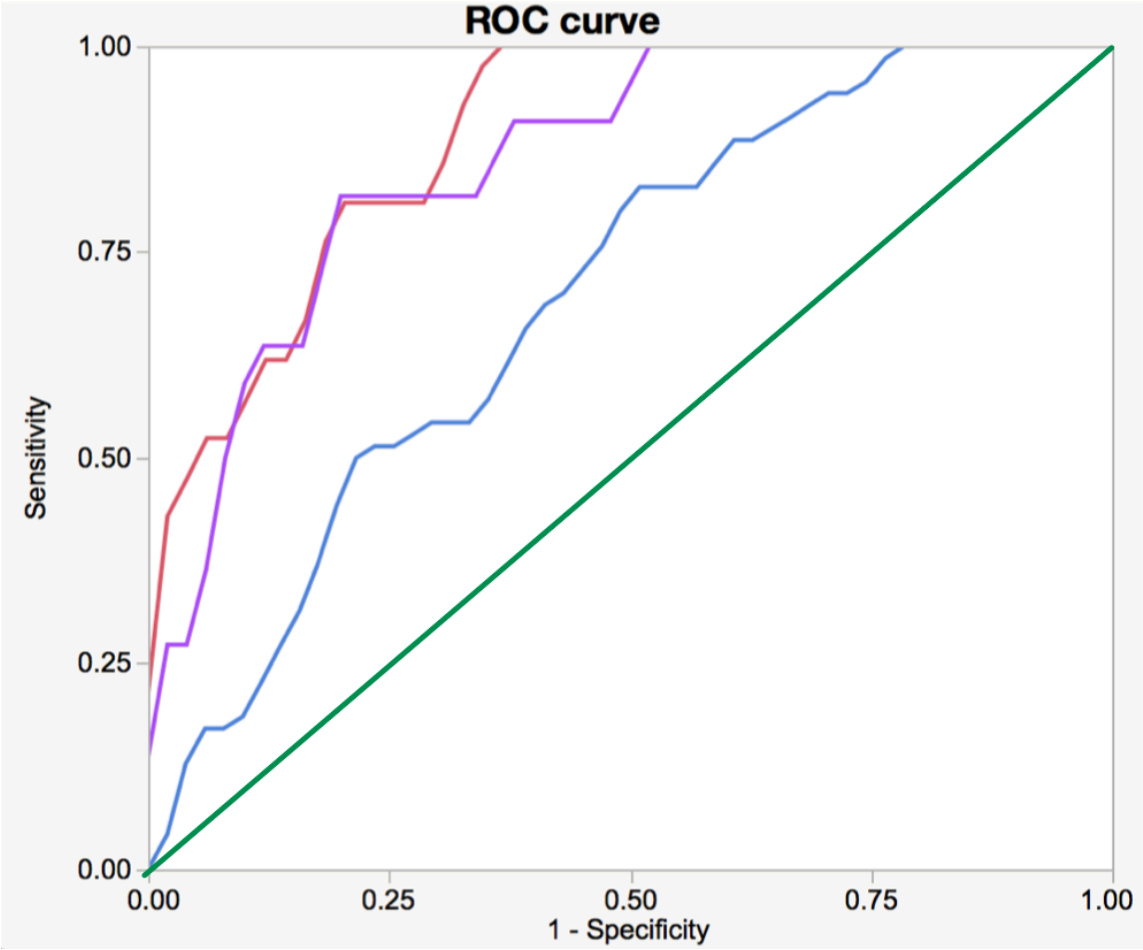


Figure 3-12 ROC curves for TBI (red), PTSD (blue) and TBI+PTSD (purple). The largest area under the curve was achieved for the model of TBI.

We further used the same regression analysis to develop models for PTSD/TBI, TBI+PTSD/TBI and TBI+PTSD/PTSD prediction. ROC analysis revealed the AUC for as follows: For PTSD/TBI the AUC was 0.771 (95% CI [0.646–0.897], $p < 0.05$). For TBI+PTSD/PTSD the AUC was 0.867 (95% CI [0.755–1.000], $p< 0.05$). No model could be deployed for TBI+PTSD/TBI as no individual lipid species passed significance test during linear regression. The ROC curves for each model are displayed in Figure 3-13. Optimal sensitivities with specificity of at least 80% predicted probabilities are shown in Table 3.3 as well. The highest sensitivity and specificity was achieved with the model for TBI+PTSD/PTSD. ROC tables can be found in the Appendix, Chapter 3, Section 2.

	*Model	Sensitivity	Specifity	# R^2
PTSD/TBI	LPC(o-18:0) LPC(20:3) LPE(18:1)	63%	80%	0.268
TBI+PTSD/TBI	-	NA	NA	NA
TBI+PTSD/PTSD	PI(36:1) PI(38:4) PE(42:6)	91%	80%	0.458

Table 3-3 Optimal sensitivities with specificities of at least 80% for the various diagnostic models*.

*Calculations based on predicted probabilities form binary logistic Regression.
Represents Nagelkerke R^2

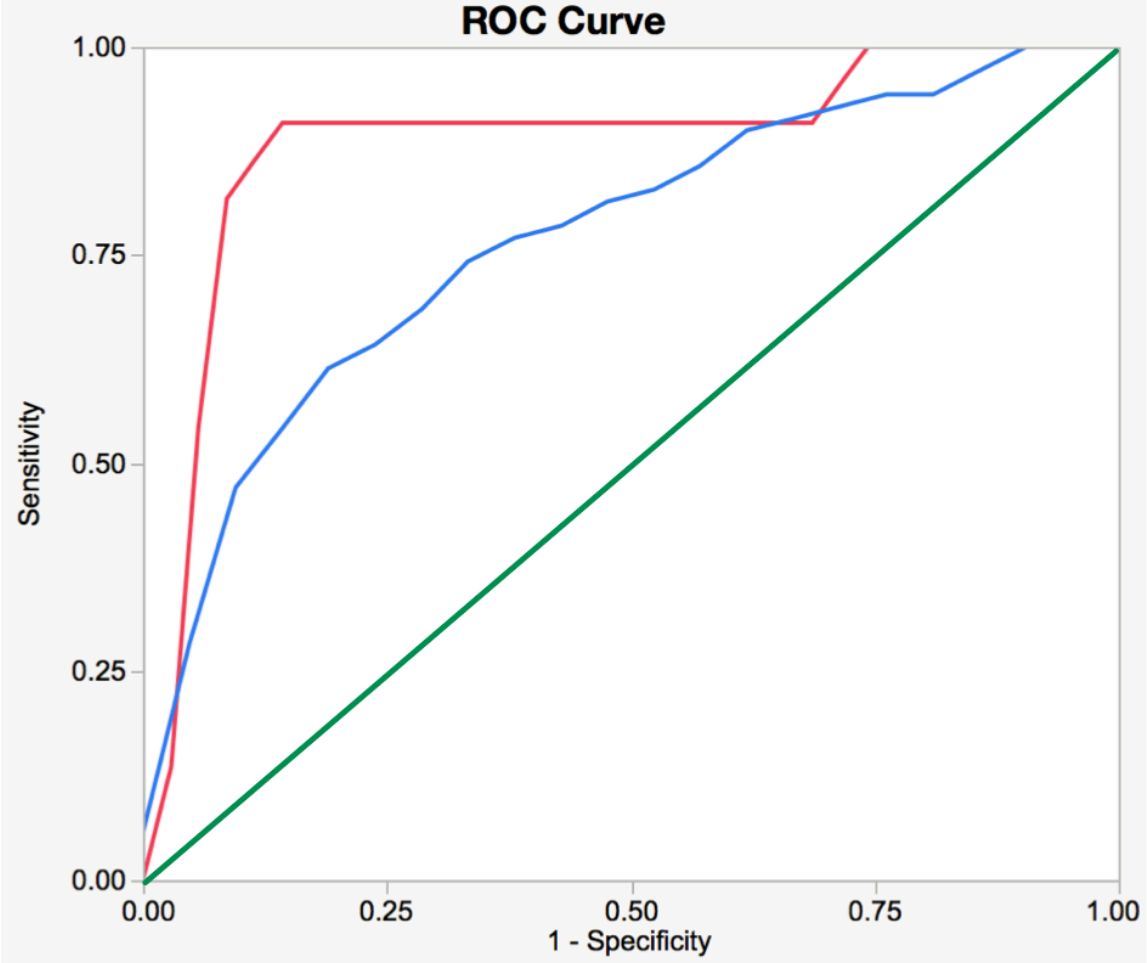


Figure 3-13 ROC curves for TBI+PTSD/PTSD (red) and PTSD/TBI (blue). The largest area under the curve was achieved for the model of TBI+PTSD/PTSD.

Plasma profiling of total PC, LPC, SM, PE, LPE, and PI in plasma of mild, and moderate to severe, PTSD subjects

The above analysis used a dichotomous grouping to differentiate controls from the diagnostic groups (exhibiting TBI, yes or no; PTSD, yes or no; or having both, yes or no), using measurements described in the Methods section. However, for PTSD, the PCL-M test allows for a severity screen by using the continuous variable scores in the test. As such, we were able to analyze PTSD subjects separately according to their PCL-

M scores in regards to PTSD severity. Subjects with 0 to 34 points were grouped as controls, 35 to 43 as mild PTSD and >44 as moderate-to-severe PTSD. Overall, total LPC, SM, LPE, and PI were decreased ($p < 0.05$) in the plasma of subjects with moderate-to-severe PTSD. There were no significant differences in total PC ($p = 0.052$) or total PE ($p = 0.063$) levels in the plasma of PTSD subjects (mild or moderate-to-severe), compared with controls, based on post hoc analyses ($p > 0.05$). Figure 3-14 shows total levels (mean ± SD) of PC, LPC, PE, LPE, SM, and PI for subjects with mild PTSD (PCL-M, 35-43) and moderate to severe PTSD (PCL-M > 44) as a percentage of the control group (PCL-M, 0-34). Compared with the control group, total LPC was decreased by 22% for the moderate-to-severe PTSD group. For total SM, the moderate-to-severe PTSD group was significantly decreased by 17%, 22% for LPE and for 30% PI. No significant differences were found in LPC, SM, LPE, and PI levels between mild PTSD and the control group.

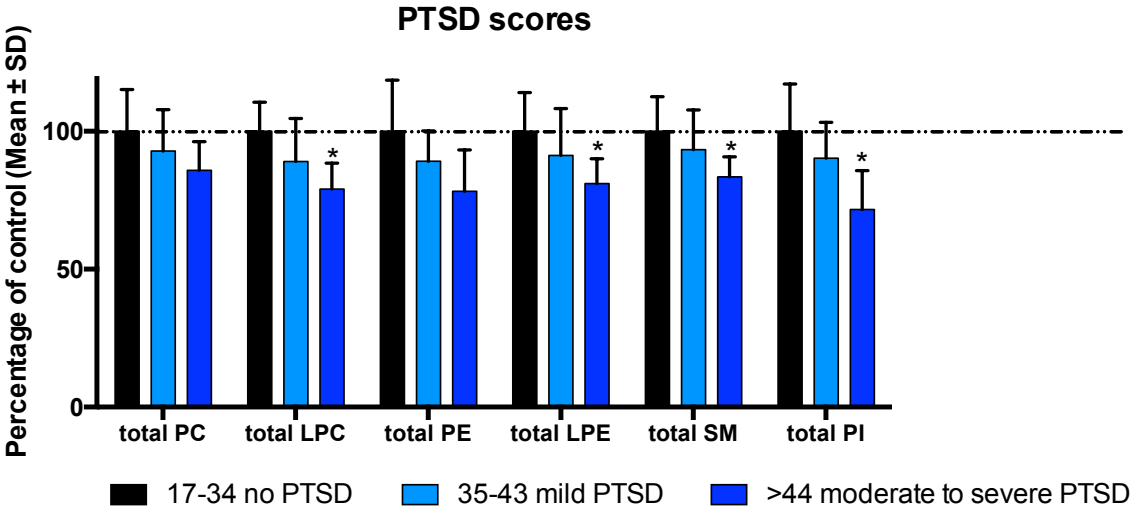


Figure 3-14 Significant changes in total plasma phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), lysophosphatidylcholines (LPC), sphingomyelin (SM), and lysophosphatidylethanolamide (LPE) in human subjects with post-traumatic stress disorder (PTSD), represented as the mean, and standard derivation (SD), as a percentage of controls. Individual molecular species of

PC, PE, PI, LPC, SM, and LPE quantified by liquid chromatography/mass spectrometry were summed after LipidomeDB analyses to generate total lipid levels. Lipid species were investigated in groups of PTSD severity, based on mixed linear modeling regression (*$p < 0.01$).

3.4 Discussion

Neurobiological changes associated with mTBI and PTSD can impact service members' well-being, long after the primary insult, causing lasting neurological dysfunction and long-term health consequences [309]. Despite the well-studied chronic impact of both TBI and PTSD, diagnostic biomarkers are not available at chronic post-injury time points. Given the complexity of the underlying pathology, unbiased omic approaches, such as lipidomics are needed to dissect the heterogeneous clinical presentations of these illnesses. Brain PL disturbances have previously been detected in the CSF of TBI subjects [310], suggesting that alterations in the brain can be detected in peripheral biofluids. In fact, studies have shown that certain PLs, particularly those that contain PUFA may be transported from blood to brain, where they are used for various functions (e.g. membrane repair, lipid mediators, etc.) [311]. Phospholipid disturbances were observed in the brain of a TBI mouse model, and showed that major PL classes (in particular PUFA-containing PLs, DHA, and ePLs) were significantly altered three months post-injury [304]. Therefore, we examined PL changes in soldiers with mTBI and PTSD, particularly in relation to the degree of unsaturation to determine if blood PL levels are altered at a chronic timepoint.

The ApoE protein plays an important role in regulating transport and homeostasis

of various lipids, including triglycerides, PL, and fatty acids, in both the periphery and the brain and is known to facilitate transport of these lipids from the blood into the brain. Production of the ApoE protein is controlled by the APOE gene, and three common isoforms of APOE are found in humans, encoded by the alleles ε2, ε3 and ε4 [312]. Whilst the distribution of ApoE among lipoproteins is influenced by the apoE allele (apoE4 has a preference for large, triglyceride-rich VLDL particles; apoE3 and apoE2 associate with the small, phospholipid- rich HDL), the effect it has on circulating plasma PL is not well understood[305]. Yet, characterization of various PL classes (PC, PE, PI and SM) in plasma from young (2month) and old (12month) human APOE ε2, ε3, and ε4 knock-in (KI) mice showed decreased total PE and increased SM levels in young APOE ε2 compared to APOE ε4 animals. Furthermore total SM levels were increased in APOE ε2 compared to ε3 in 12month old mice[313]. A clinical study, which investigated the consumption of 1.1.g/day of DHA for six week showed that the rise in DHA level was 60% lower in plasma triglycerides APOE4 carriers as compared to the non-carriers[314]. Overall, effects on APOE on circulating plasma PL needs further investigation and we therefore stratified our analysis by apoe ε4 carrier status.

Furthermore, clinical studies suggest poor long-term cognitive and functional outcomes in TBI patients with the APOE ε4 compared to those without the ε4 allele [74,315]. In another human study that included participants with combat trauma sustained during the Vietnam War, Lyons et al investigated apoE ε4 (-) and ε4 (+) genotype interaction with level of combat exposure to predict the number of PTSD symptoms given exposure to combat trauma [316]. Their findings suggest that the APOE ε4 allele is a risk factor for the negative consequences of combat exposure. At the blood-brain barrier (BBB), fatty acids

are taken up by brain astrocytes and then utilized as an energy source and for lipid synthesis [317]. Since APOE represents an important mediator between injury and lipid abnormalities, we investigated a possible effect of the APOE ε4 carrier status on PL profiles, which are discussed below.

Our results revealed a significant decrease in PL levels (total PC, LPC, SM, PE, LPE, and PI) in active-duty soldiers screened for mTBI, PTSD, and mTBI with comorbid PTSD compared to healthy controls. Although PL plasma profiling in humans has not been done for chronic stages of the injury, clinical studies by Pasvogel et al. showed that subjects with severe TBI have increased levels of PC and PE in the CSF in those who died days following injury compared to those who survived [299]. Furthermore, studies show elevated levels of serum cholesterol, LDL, triglycerides (TG), and decreased high-density lipoprotein (HDL)[300–302] in subjects with chronic PTSD. However, further investigation of lipid transport is necessary in order to determine how peripheral lipid changes are associated with mTBI and PTSD pathogenesis.

The brain can synthesize SFAs and MUFAs, whereas PUFAs are largely acquired through the periphery due to the low capacity of the brain to synthesize these *de novo* [318]. Therefore, we examined the degree of unsaturation of PL species to determine if PUFA containing PLs were differentially affected in response to injury. The results indicated that for LPC, PE, and LPE species, there was no effect on the degree of unsaturation. LPC, PE, and LPE containing SFA, MUFA, and PUFA species were all decreased in TBI, PTSD, and/or TBI+PTSD compared to controls. In addition, PC and PI, SFA and PUFA containing species were decreased in all three groups compared to controls. However, MUFA containing PC (ePC [34:1], PC [34:1], ePC [36:1] and PC [36:1]) and

PI (PI [34:1] and PI [36:1]) species were lower in the TBI and TBI+PTSD groups only, whereas levels of PTSD were not affected.

As ApoE is involved in PUFA transport we examined PLs and their subcategories by APOE ε4 carrier status within our study population. An examination of APOE genotype on the degree of unsaturation revealed that subjects carrying the APOE ε4 allele showed differences of PL profile levels for TBI, PTSD, and TBI+PTSD compared to APOE ε4 non-carriers. For PE, we found a significant interaction between APOE ε4 and diagnosis on SFA and MUFA containing PE species. APOE ε4 non-carriers showed decreases in SFA and MUFA containing PE species for TBI, PTSD, and TBI+PTSD groups compared to controls. Among APOE ε4 carriers, there were no significant differences between the TBI and the PTSD group for SFA and MUFA containing PE species. However, the differences in SFA and MUFA species were also significant among the APOE ε4 carriers for the comparisons between the TBI+PTSD group and the controls. There was a significant interaction between the presence of APOE ε4 allele and diagnosis of TBI+PTSD on MUFA-containing LPC and LPE species. For MUFA-containing LPC and LPE species, APOE ε4 non-carriers showed a significant decrease in lipid levels for TBI, PTSD, and TBI+PTSD as compared to controls, whereas APOE ε4 carriers only showed decreases for TBI+PTSD subjects compared to controls.
We also observed a significant effect of APOE ε4 together with diagnosis on PUFA-containing LPE species. APOE ε4 non-carrier showed significant decrease in LPE levels in TBI, PTSD, and TBI+PTSD subjects, whereas levels of LPE were elevated in APOE ε4 carriers with PTSD and in the TBI+PTSD group. These observations might be of particular interest, as PUFAs are primarily acquired through the periphery into the brain.

We hypothesize that carrying the APOE ε4 allele leads to a reduced transport of PUFAs to the brain via the apolipoproteins, which might explain why plasma PUFA levels in mTBI subjects were not statistically different from control subjects; not meeting the brains higher energy needs. However, we did not observe a significant APOE ε4 effect on PUFAs of the other PL classes we investigated.

Phospholipids and their metabolites activate downstream inflammatory cascades, which contribute to the immune and inflammatory balance within the CNS and the periphery. Of particular note, DHA and AA are the most abundant ω-3 and ω-6 PUFAs in the brain. However, also in plasma, brain, and CSF, DHA is a precursor of the anti-inflammatory mediators, resolvins, and neuroprotectins, whereas AA is a precursor of pro-inflammatory lipid mediators, such as prostaglandins and leukotrienes [319,320]. Studies by Vandal et al., showed a lower BBB transport of DHA in mice homozygous for APOE ε4 relative to APOE ε4 non-carriers [321]. We therefore examined ratios of AA to DHA containing PLs and found a significant decrease in ratios in TBI+PTSD subjects for PC and LPC, and PE for TBI and TBI+PTSD subjects. AA containing species are preferentially being metabolized over DHA, which might be further propagating inflammation already associated with TBI and PTSD. This might indicate increased DHA over AA, leading to the generation of anti-inflammatory neuroprotectins in order to resolve ongoing inflammation. We also examined the effect of APOE ε4 carrier status on the PL lipid species and observed a significant effect on the ratios of AA to DHA containing PC, PI, and LPC classes. Among APOE ε4 non-carriers, for PC, PI, and LPC, there was a significant decrease for the ratios of AA to DHA with respect to different diagnostic categories (PC; TBI+PTSD, PI; TBI, PTSD and TBI+PTSD, LPC;

TBI+PTSD) compared to controls. However, within APOE ε4 carriers, lipid level did not differ from control subjects. Moreover, we observed a significant increase of the ratio in LPC species in mTBI and PTSD subjects in APOE ε4 carrier subjects. As the brain needs certain lipids, such as PUFA and DHA, there might be an increased need for lipids after injury for repair and other related functions [322–324]. Given the role of APOE in transport of PUFA and the observed deficiencies in transport of these lipids as a result of the APOE ε4 allele (as described above), it is possible that the APOE ε4 carrier status could further exacerbate the effects of injury/disease.

In recent years, the question of how ApoE regulates DHA metabolism and transport has arisen, as low plasma DHA concentration has been associated with cognitive decline in otherwise healthy elderly individuals and patients with Alzheimer's Disease [325–328]. Moreover, studies have suggested that dementia patients who do not have the APOE ε4 allele benefit from DHA supplementation in contrast to APOE ε4 carriers [329–331]. We observed an increased ratio of AA to DHA in APOE ε4 carriers, which might suggest impaired transport of these species to the brain resulting in an accumulation of DHA in the plasma. However, further investigation of lipid transport in relation to the APOE genotype and mTBI/PTSD is required to support this hypothesis.

In order to investigate which subtypes of PLs could be useful in the future for diagnostic purposes, we evaluated ether PLs and platelet-activating factors (PAFs). These PLs promote inflammation and increased BBB permeability after brain injury and are produced by hydrolysis of alkyl ether ePCs [332,333]. We observed differences in total ePC in plasma of subjects with TBI, PTSD, and TBI+PTSD. In general, ePCs are abundant in PC and PE species. Levels of ePC were significantly decreased in PTSD and TBI+PTSD

groups relative to controls. In the current study, we also found a significant APOE ε4 interaction effect with ePC and ePE species. In APOE ε4 non-carriers, ePC lipid levels were significantly decreased in TBI, PTSD, and TBI+PTSD subjects, whereas APOE ε4 carriers in all groups showed similar levels to control subjects. Among APOE ε4 non-carriers, ePEs were significantly decreased in TBI, PTSD, and TBI+PTSD subjects, whereas APOE ε4 carriers showed only a significant decrease for TBI+PTSD. As such, measurement of ePE levels combined with APOE genotyping might be useful to evaluate as a differential diagnostic tool in future studies.

We also performed a preliminary ROC analysis via a model established through linear regression containing significant individual lipid species for each diagnostic group. The model revealed to have highest sensitivity and specificity for TBI/Control, whereas PTSD/Control performed poorly. The model for TBI+PTSD/Control achieved similar sensitivity at the given 80% specificity mark as TBI/Control. For TBI+PTSD/TBI no model could be established, whereas PTSD/TBI performed poorly. The model for TBI+PTSD/PTSD however achieved good sensitivity at 80% specifity. It is important to note that this analysis has to be extended and further investigation is needed to develop a final model for prediction of diagnostic groups.

In our final analysis, we examined differences in total PL levels in subjects by PTSD severity. This allowed us to observe if our previously investigated lipid species correlated with the established PCL-M questionnaire. Subjects were subdivided based on PCL-M scores to investigate if lipid levels correlate with PTSD severity. In subjects with moderate-to-severe PTSD (PCL-M $\geq$ 44), we found a significant decrease of LPC, LPE, SM and PI levels. In contrast, we did not find significant differences between controls

and subjects with mild PTSD (PCL-M: 35-43), suggesting that dysfunction in lipid homeostasis only occurs as a result of more severe PTSD.

Finally, in order to minimize the effect of other factors that could influence lipid levels only subject were included, who did not use lipid level altering medication. Furthermore, as metabolism can influence plasma lipid levels, a military cohort was sampled in order to provide subjects with comparable fitness level and standardized training as well as uniform diet.

3.5 Limitations

One limitation of this study is that for the subjects diagnosed with mTBI, an accurate estimation of the duration of time between the injury and the blood collection is unavailable, though this is a common issue in cross-sectional study designs.
Another limitation of the current study was sample size. Although sufficient size was achieved to power the conducted analyses, sample sizes are small for the APOE ε4 carrier analysis (n=5 for APOE ε4 carriers in the mTBI and mTBI+PTSD group). This was mainly due to the disproportionately low number of ε4 allele carriers within our sample, which is reflected in the general population. Larger samples sizes are of course desirable; however studies such as these are very difficult to coordinate and, given their importance to the research community, we, along with others in the field [334–336], consider their presentation of critical importance for validation and replication in the community. Future studies should emphasize collecting data on larger numbers of military service members in order to explore potential interactions and genetic mediators of combat-

related PTSD and mTBI. Moreover, this will allow for analysis of specific PTSD subgroups based on symptom clusters. Furthermore, diagnostic categories were established using screening instruments, not provider diagnoses. The military health care system is developing and implementing tools with mechanisms in order to track patients through all stages of their care. Advancements, such as these, will be invaluable for addressing many caveats beyond the scope of this current work. Nonetheless, this study, which enabled work with an active duty population, addresses a chronic perspective of TBI/PTSD biomarker research, which has not been done before in this format.

3.6 Conclusion

The current study performed lipidomic characterization of different plasma PL classes in active-duty soldiers with a history of mTBI, PTSD, and comorbid mTBI and PTSD, in order to evaluate if secondary injury damage and traumatic stress have an impact on PL levels. We successfully showed a characterization of PLs in plasma, associated with these diagnostic categories. In addition, we revealed that the APOE genotype, specifically ε4 carrier status, may be associated with plasma PL levels. Despite the extensive research of APOE and lipid metabolism in TBI, more studies are needed to identify the underlying mechanisms in order to develop a biomarker panel that has the sensitivity and specificity to diagnose mTBI and PTSD. We expect that additional profiling of proteins, other lipid species, and genetic interactions can lead to a successful identification of diagnostic and prognostic biomarkers. However, findings need to be replicated in larger cohorts to determine the potential utility of PLs as biomarkers.

Chapter 4 Mild TBI results in a long-term decrease in circulating phospholipids in a mouse model of injury

4.0 Summary

In this chapter I continued the omics analysis in an animal model of mTBI that has been established and characterized at the Roskamp Institute. Neurophysiological and neurological dysfunction is usually experienced for a short period of time in patients with mild traumatic brain injury (mTBI). However, around 15% of patients continue to exhibit symptoms months after TBI. Phospholipid (PL) changes have been observed in plasma from mTBI patients at chronic stages, suggesting a role in TBI pathology. We examined long-term plasma phospholipid profiles in a mouse model of mTBI to validate its value in reproducing PL changes observed in mTBI patients. Plasma samples were collected at an acute time point (24 hrs post-injury) and at several chronic stages (3, 6, 12 and 24 months post-injury) from injured mice and sham controls. Phospholipids were identified and quantified using liquid chromatography/mass spectrometry (LC/MS) analysis. In accordance with our human data, we observed significantly lower levels of several major PL classes in mTBI mice compared to controls at chronic time points. Saturated, monounsaturated and polyunsaturated fatty acids (PUFAs) were differently regulated over time. As PUFA levels were decreased at 3 months we measured levels of malondialdehyde to assess lipid peroxidation, which we found to be elevated at this timepoint. Ether containing PE species were elevated at 24 hrs post-injury and decreased

relative to controls at chronic stages. Arachidonic acid (AA) and docosahexaenoic acid (DHA) containing species were significantly decreased within all PL classes at the chronic stages. Our findings are similar to the changes we observed in PL levels in human mTBI subjects (Chapter 3). Furthermore, I measured LRG1 levels in this mTBI model to investigate if the increases I observed in humans (Chapter 2) could be observed, and found significant elevations at specific timepoints in mTBI versus control mice. Despite the significant disabilities evident at chronic stages after TBI, little attention has been paid to the identification of biomarkers at chronic stages post-injury. Our study shows biochemical abnormalities that persist long after the initial injury and provides support for our human study. The validation of this model will further help with the investigation of preclinical studies.

4.1 Introduction

We previously discussed how mild TBI typically results in the lack of structural abnormalities using conventional brain imaging techniques, with most patients recovering after injury. Yet, a significant number of individuals (10-15%) experience persistent post-injury symptoms [337,338]. Very few studies to date have demonstrated the chronic effects of mTBI on neuropathological outcome in humans and in animal models [339,340].

It is challenging to study mTBI in humans, due to the heterogeneity, as well as the lack of initial appearance of severity, which therefore can lead to a lack of medical attention in the acute aftermath. The type of TBI, patient demographics, time post injury at the time of sample collection and genetic predisposition can all influence sensitivity and specificity of possible candidate biomarkers. Therefore the use of animal models presents an opportunity to control for the mentioned confounding factors and will lead to better understanding of physiological and molecular changes from the acute phase of minutes to hours, up to sub-chronic and chronic stages (days to months). The use of laboratory models of relevance to human TBI enables biomarker profiling over extended periods post-injury, which would be very difficult to assess in human patient populations.

In this chapter, we used the previously described liquid chromatography/mass spectrometry (LC/MS) analyses to investigate long-term plasma PL profiles and also to assess changes in LRG1 concentration in a closed head injury (CHI) mouse model of mTBI in order to determine whether this model captures the lipid and protein changes observed in human mTBI subjects.

The mouse model described here has been previously characterized [341,342] (Mouzon *pers. comm*.). Mouzon and his colleagues at the Roskamp Institute specifically developed a CHI model of mild TBI to address this understudied area of TBI research. Other models such as the fluid percussion injury (FPI) or controlled cortical impact (CCI) require a craniectomy and are relatively severe by comparison. It has been previously demonstrated that these invasive methods can confer profound behavioral and proinflammatory damages [343]. The described CHI model has been characterized over an extensive period of time and the group has shown that mice exposed to a single mTBI had subtle and transient behavioral and immunohistochemical abnormalities with long lasting pathology. Firstly, behavioral testing during the first 2 weeks post injury showed transient deficits in motor function and spatial memory in mTBI mice. Furthermore, at 24hrs, reactive astrocytosis and sparse APP-immunoreactive axonal pathology in the corpus callosum were observed [341]. Secondly, investigation of this group was extended to several chronic timepoints (6 and 12 month timepoints published; 3 month and 24 month unpublished). The group showed that cognitive performance returned to normal by 6 months after a single mTBI [342]. These observations are consistent with findings in human patients in which those suffering from mild head injury often return to their pre-concussive cognitive status within a week [344,345]. Yet, injured animals showed neuroinflammation and white matter loss (thinning of the corpos callosum (CC)), which peaked at 6 months and remained static at the 12 months timepoint compared to controls [342]. The neuropathology we see in these mice is consistent with some aspects of human TBI as confirmed by our clinical collaborator Dr. William Stewart in Glasgow, Scotland, who is a clinical neuropathologist specializing in TBI. Therefore, we used our

phospholipid analysis platform to investigate plasma samples collected from these animals at 24hrs, 3, 6, 12 and 24 months post injury. We hypothesized that changes in the PL of mice could reflect our previous findings in human subjects.

4.2 Materials and Methods

Animals

Male, C57BL/6J mice (10 weeks, 24–30g, Jackson Laboratories, Bar Harbor, ME) were singly housed under standard laboratory conditions (23°C±1°C, 50%±5% humidity, and 12 h light/dark cycle) with free access to food and water throughout the study. We used a standard diet (ENVIGO, IN) that contains a standard mixture of fatty acids (both omega-6/omega-3, SFA (0.8%), MUFA (1.1%) and PUFA (2.9%)). Mice were allowed to adapt to the vivarium for 1 week prior to experimental procedures. All procedures involving mice were performed under Institutional Animal Care and Use Committee approval and in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Injury protocol

Mice were injured via a CHI mTBI model as previously described [341,342]. Briefly, mice were anesthetized with 1.5 L/min of oxygen and 3% isoflurane and their heads shaved; each mouse was transferred into a stereotaxic frame (Stereotaxic, Stoelting, Wood Dale, IL) mounted with an electromagnetic (EM) controlled impact device (Impact OneTM Stereotaxic Impactor, Richmond, IL). The injury was triggered above the sagittal

suture midway using the myNeuroLab controller at a strike velocity of 5 m/sec, strike depth of 1.0mm, and dwell time of 200 ms, a paradigm specifically developed to avoid fractures and bleeds to allow correlation with human mTBI. Based on the manufacturer's instructions, the force applied to the mouse head at the time of impact is 72N under these conditions. At the end of the procedure, each animal was allowed to recover and was then returned to its home cage. Control animals underwent the same procedures and anesthesia of the same duration, but did not receive a hit, in order to control for the effects of anesthesia.

Injury groups and schedule

For the lipidomic analyses, a total of 40 mice were randomly assigned to one of two groups - single injury (mTBI) or single sham controls (control: anesthesia alone matched for the anesthesia duration of injured mice). Samples were collected at five different timepoints - 24 hours and 3, 6, 12 and 24 months post injury (n=8 per timepoint, comprising 4 mTBI and 4 control mice).

Sample preparation

To obtain blood specimens suitable for measurement of plasma phospholipid analysis, animals were lightly anesthetized with isoflurane prior to euthanasia, and approximately 500µl of blood was withdrawn into EDTA capillary tubes by cardiac puncture to avoid coagulation. Protease and phosphatase inhibitors were added as additional preservatives. Blood was then centrifuged at 5,000 x g for 3min to avoid shearing of cells [253,346]. Plasma was collected and stored at -80°C until lipidomic analysis.

Figure 4-1 shows the outline of our experimental schedule. On the day of euthanasia, all mice in that cohort were euthanized randomly (control or injured) between 9am and 2pm to avoid any bias due to collection time. All mice were non-fasting at the time of euthanasia; it has been shown that fasting has no effect on changes in PL levels [347]; given the standardization of diet and treatment/handling of all animals we expect PL levels to be stable across groups apart from any influence of mTBI.

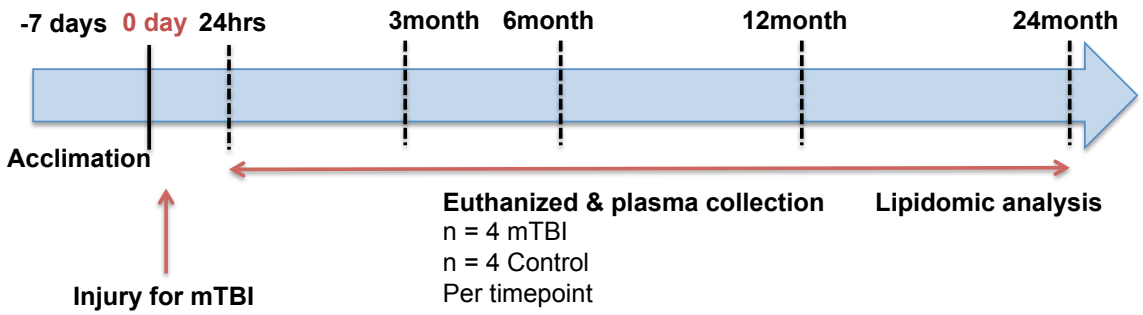


Figure 4-1 Outline of experimental schedule

Thiobarbituric Acid Reactive Substances (TBARS) ELISA

For the 24hrs and 3 months timepoint, lipid peroxidation was assessed via a TBARS ELISA (Cayman Chemical, MI). As lipid peroxides are unstable a number of compounds are formed, including malondialdehyde (MDA). Therefore, the level of peroxidation is measured by the occurrence of MDA. The ELISA was performed following manufacturer's instructions. A MDA-thiobarbituric Acid (TBA) adduct was formed by the reaction of MDA and TBA under high temperature (90-100°C) and acidic conditions, and measured colorimetrically at 530-540 nm.

Leucine-rich alpha-2 glycoprotein 1 ELISA

LRG1 concentrations were determined using a competitive inhibition enzyme-linked immunoassay (ELISA, www.mybiosource.com) applying the same principle as the human LRG1 ELISA (Chapter 2).

For both TBARS and LRG1, significance was determined by one-way ANOVA. P values < 0.05 were considered statistically significant.

Lipidomic Analysis

All samples were analyzed with the experimenter blinded to the group and time point to which each animal was assigned. Lipids were extracted from plasma (n = 4 mTBI and n=4 control for each of the 5 timepoints post injury; in total N=40) using the Folch method (Folch et al. 1957; Emmerich et al. 2015). Lipidomic mass spectrometry analysis was performed as previously described in Chapter 3.

Statistical lipid analyses

For phospholipids, statistical analyses were performed as we have previously described in Chapter 3 [228,348].

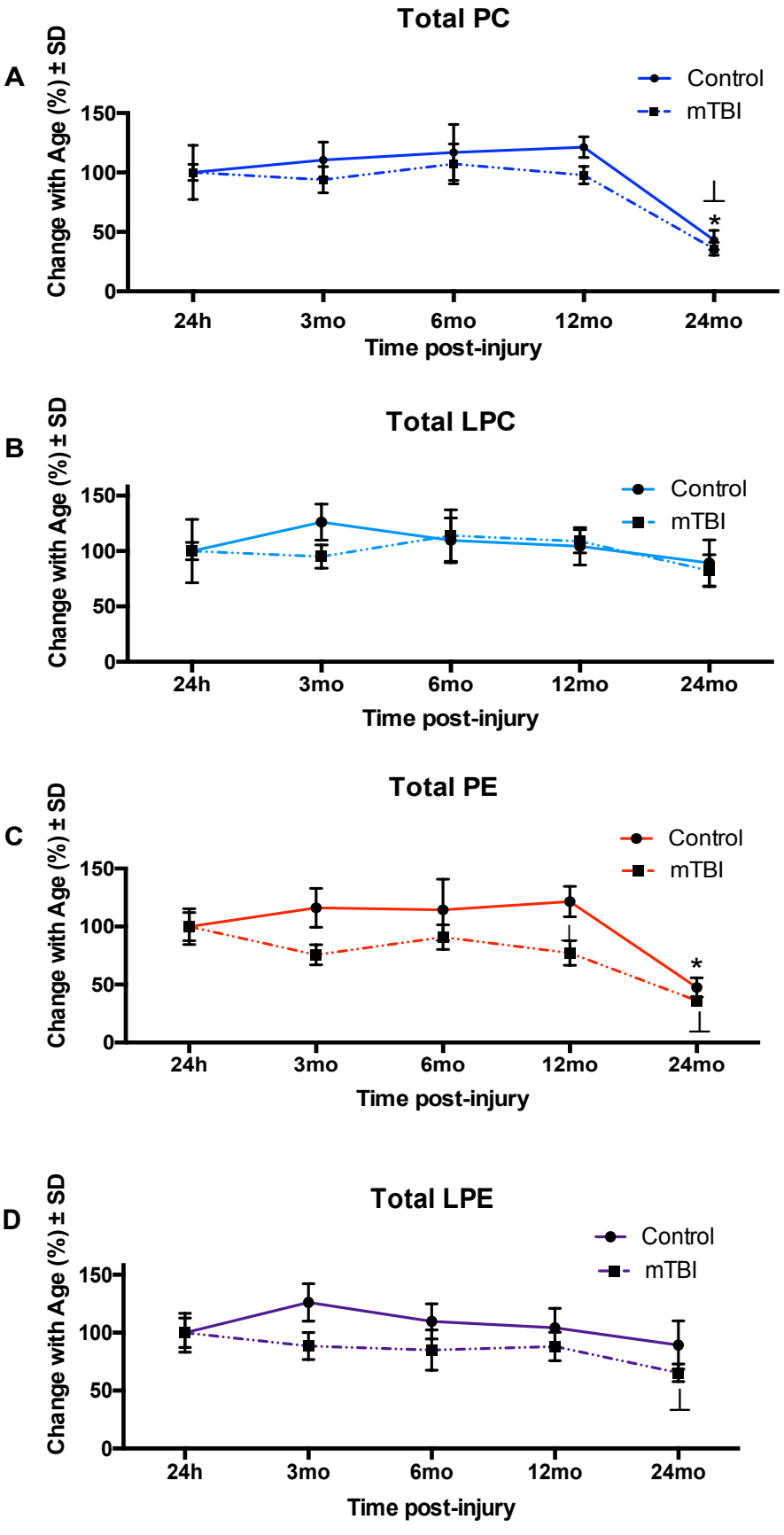
4.3 Results

We used HILIC LC/MS to identify and quantify various phospholipid classes and their molecular species.

Long-term plasma profiling of total phospholipid classes in mTBI and control animals

Our aim was to establish a temporal plasma PL profile in mTBI injured mice for comparison against matched control mice (sham). We examined PL profiles at an acute timepoint (24h) and at multiple chronic post-injury time-points (3-, 6-, 12- and 24 months post injury).

In Figure 4-2 A-F, we first show the possible effects of aging on total PL levels in control mice (solid lines) and mTBI mice (dashed lines) ranging from 24hrs to 24 months. For control mice, we observed significantly lower PC, PE, PI and SM levels at the 24 month timepoint relative to 24hrs ($p < 0.05$), but no significant differences between 24hrs and any of the other timepoints evaluated in this study. No effect of aging was observed for LPC and LPE in control mice. The mild TBI group showed as well significantly lower levels at the 24 month timepoint for PC, PI and SM. For LPC in mTBI mice, no changes were observed for all timepoints. However, in mTBI mice total LPE, reached significantly lower levels at 24 months compared to 24hrs. For total PE, the 12 and 24 month levels were decreased compared to 24hrs.


A
Total PC
Change with Age (%) ± SD
Control
mTBI
150
100
50
0
*
24h
3mo
6mo
12mo
24mo
Time post-injury
B
Total LPC
Change with Age (%) ± SD
Control
mTBI
150
100
50
0
24h
3mo
6mo
12mo
24mo
Time post-injury
C
Total PE
Change with Age (%) ± SD
Control
mTBI
150
100
50
0
*
24h
3mo
6mo
12mo
24mo
Time post-injury
D
Total LPE
Change with Age (%) ± SD
Control
mTBI
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Time post-injury

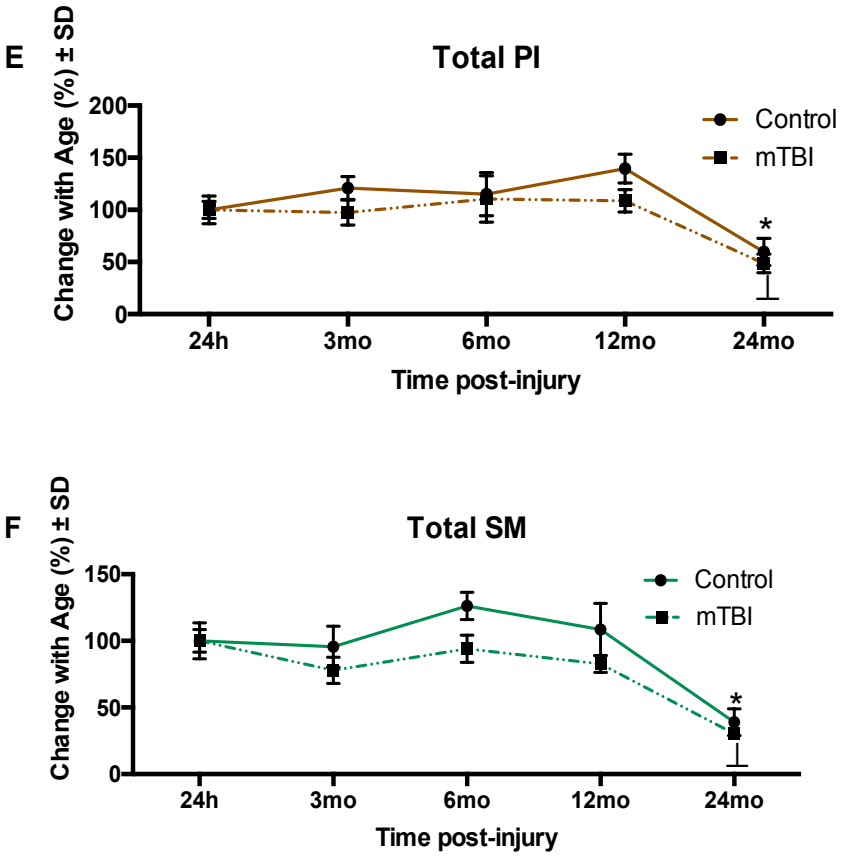
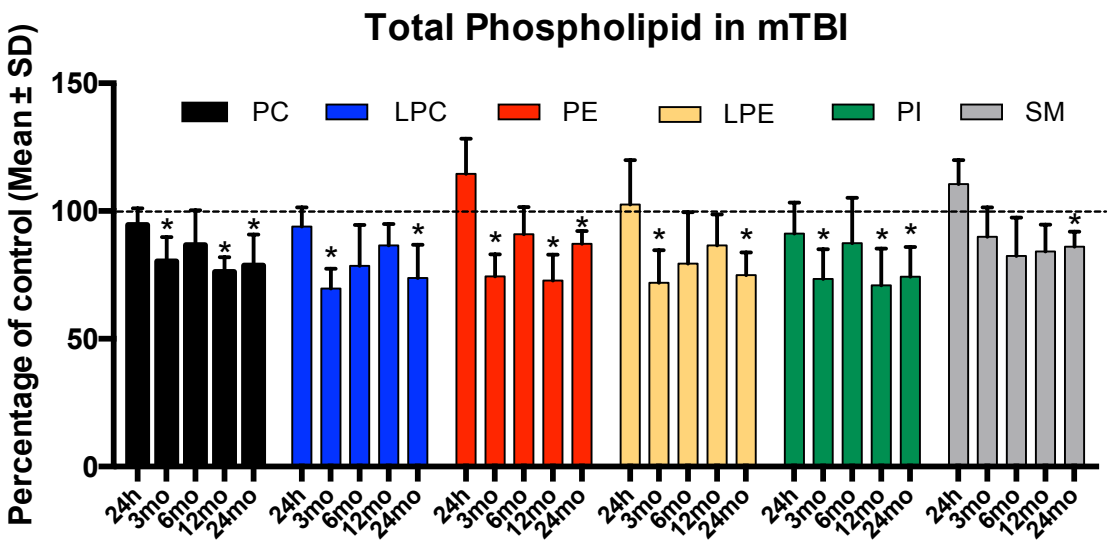


Figure 4-2 A-F. Effect of aging on total plasma PC, PE, PI, LPC, SM and LPE in control and mTBI animals, represented as the mean ± SD as a percentage of their value at the 24hrs timepoint. Individual molecular species of PC, PE, PI, LPC, SM and LPE quantified by LC/MS were summed after lipidomeDB analyses to generate total lipid levels. Levels of each total lipid class were analyzed in relation to their respective 24hrs timepoint (100%). For control mice, total PC, PE, PI and SM were significantly decreased at 24 months post injury. No significant change was observed for any other timepoints or for total LPC and LPE (* denotes $p<0.05$ in control, solid line). mTBI mice behaved in a similar fashion to their control counterparts for total PC, LPC, PI and SM. However, for total LPE, mTBI mice reached significantly lower levels at 24 months compared to 24hrs. For total PE, the 12, and 24 month values were decreased compared to 24hrs (⊥ denotes to $p<0.05$ in mTBI, dashed line).

Next, we analyzed plasma PL changes for mTBI vs. control animals at each timepoint. Total concentrations of each PL class are shown in Figure 4-3. Overall, plasma levels of several major PL classes were decreased in mTBI compared to control mice at several chronic post-injury timepoints. More specifically, relative to control mice, total PC, LPC, PE, LPE and PI were consistently lower in mTBI mice at the post injury timepoints of 3 to 24 months, with differences being statistically significant for 3, 12 and 24 months post-injury. However, SM levels were only significantly lower at 24 months post-injury. Absolute plasma concentrations for all major PL classes in control and mTBI mice can be found in supplemental table 1 (Appendix, Chapter 4, Section 1). Figure 4-4 shows a summary figure of total lipid changes over time following head injury. Overall, while there is a small but non-significant increase at 24hrs post injury, PL decreases are prominent at 3 months post injury, which is then followed by a recovery phase at 6 months. Long-term PL changes are evident at 12 months post injury with PL further decreasing at 24 months post injury, where an additional aging effect is evident.



	total PC	total LPC	total PE	total LPE	total PI	total SM	
24h							**24h**
3mo	*p=0.03	*p=0.002	*p=0.006	*p=0.02	*p=0.03		**3mo**
6mo							**6mo**
12mo	*p=0.002		*p=0.002		*p=0.038		**12mo**
24mo	*p=0.008	*p=0.008	*p=0.026	*p=0.001	*p=0.01	*p=0.038	**24mo**

Figure 4-3 Significant changes in total plasma phospholipids in mTBI mice, represented as mean percentage of control ± SD at 24hrs, 3month, 6month, 12month and 24month post injury. Individual molecular species of PC, PE, PI, LPC, SM and LPE quantified by LC/MS were summed after lipidomeDB analyses to generate total lipid levels. The table shows the timepoints at which significance was achieved. Based on MLM regression and post hoc analysis, total PC was significantly decreased at 3 month, 12 month and 24 month post injury ($p<0.01$) compared to control mice. Total LPC ($p<0.01$) and LPE ($p<0.001$) were significant at the 3 month and 24 month timepoint. Total PE ($p<0.01$) and PI ($p<0.05$) were decreased at 3 month, 12 month and 24 month. Total SM was different between injury and control at 24 month ($p<0.01$). The table gives p values for the timepoints, where changes between mTBI vs. control mTBI vs. control were significantly different.

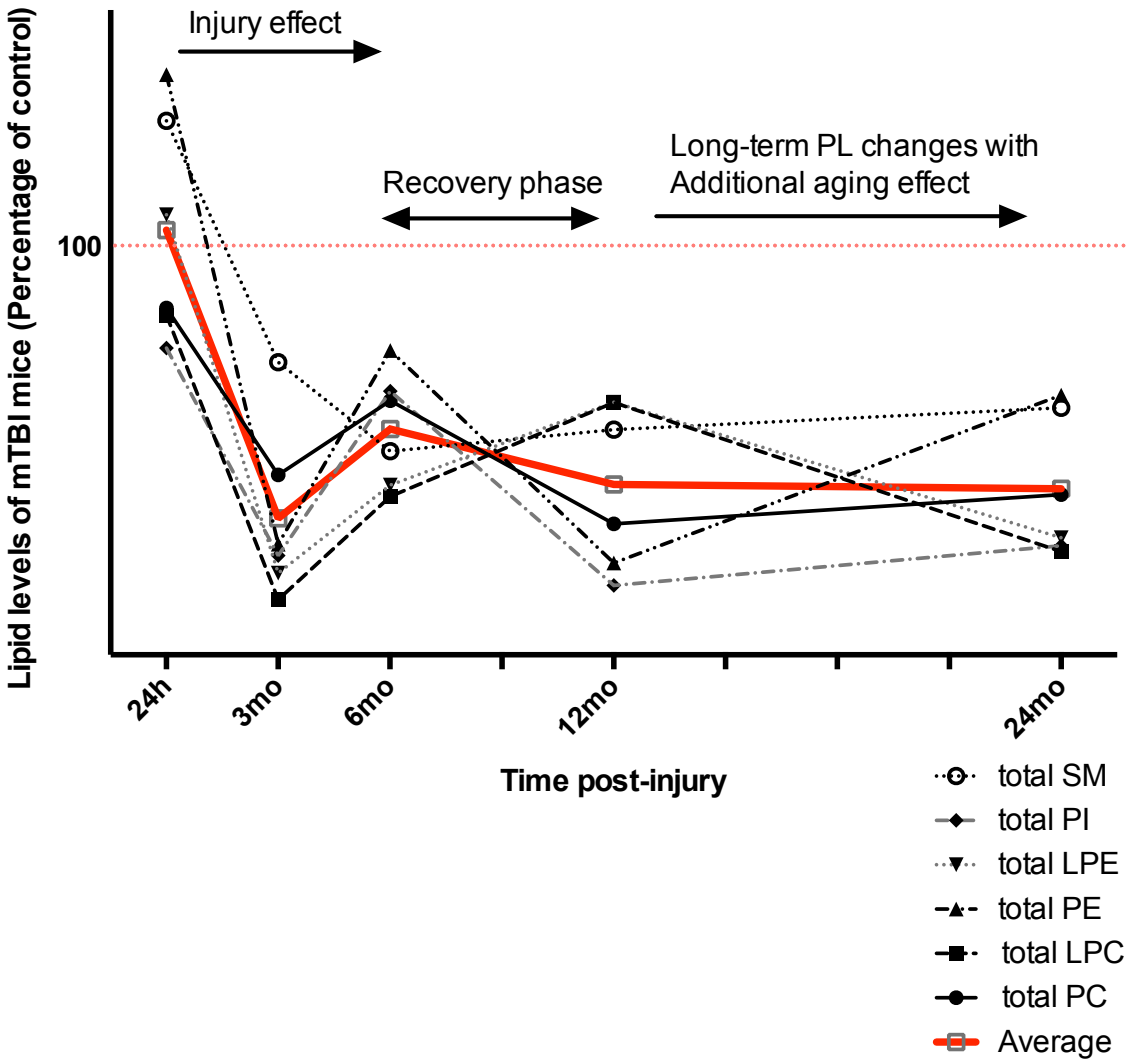
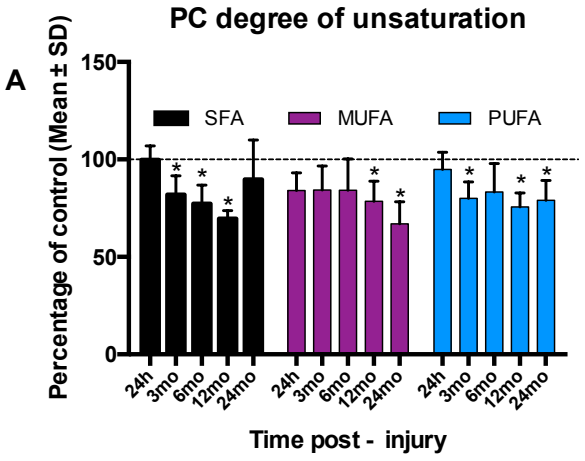


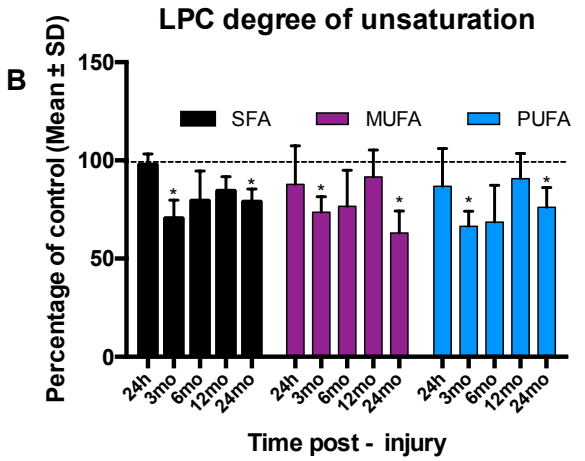
Figure 4-4 Summary figure providing an overview of our PL findings. Our overall data suggest decreases in PL at 3 months post injury, followed by a recovery phase (up to 6 months) and long-term lipid changes, which are then accompanied by aging effects. Black/grey lines indicate total changes of each PL class over time. The red line shows average changes at 24hrs, 3, 6, 12, and 24 months post injury in mTBI mice relative to controls.

Analysis of the degree of unsaturation of PL classes

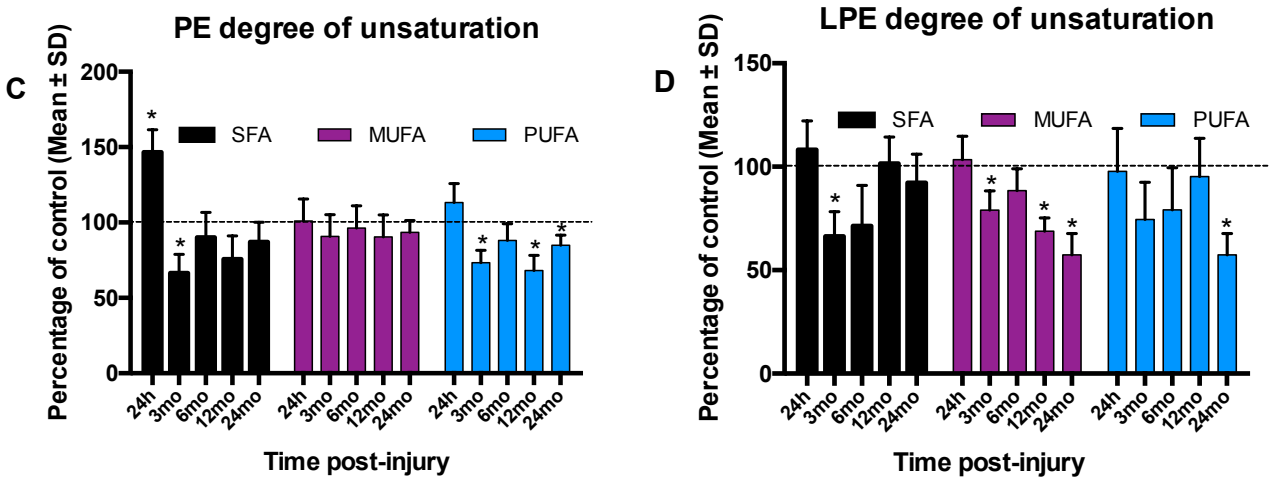
The brain can synthesize SFA and MUFA, whereas PUFA are largely acquired through the periphery due to the low capacity of the brain to synthesize these *de novo*. We therefore examined whether there was an effect of injury on the degree of unsaturation within different PL classes (Figure 4-5 A-E) at the chronic post-injury timepoints. Overall, SFA, MUFA and PUFA were decreased at several timepoints for PC, LPC and LPE in mTBI mice compared to their control counterparts. Exceptions were MUFA containing PE and PI species, which did not reach significance for any given timepoint ($p<0.05$, Figure 5C and E). However, 24h post injury, SFA containing PE species were significantly increased ($p<0.05$), whereas the degree of unsaturation of other PL classes was unaffected at this acute timepoint. Moreover, for all PL classes, PUFA-containing lipids were significantly decreased 24 months post injury.



	SFA	MUFA	PUFA	
24h				24h
3mo	*p=0.03		*p=0.006	3mo
6mo	*p=0.008			6mo
12mo	*p=0.002	*p=0.002	*p=0.002	12mo
24mo		*p=0.008	*p=0.026	24mo

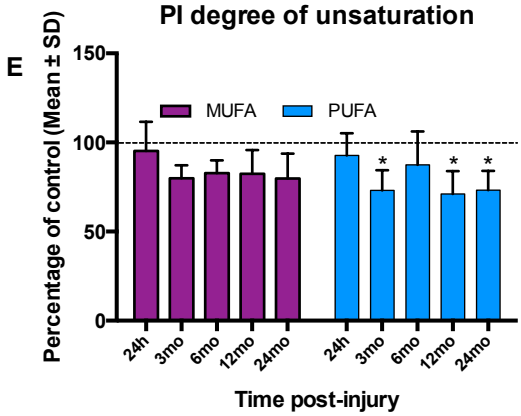


	SFA	MUFA	PUFA	
24h				24h
3mo	*p=0.002	*p=0.007	*p=0.014	3mo
6mo				6mo
12mo				12mo
24mo	*p=0.018	*p=0.002	*p=0.044	24mo



	SFA	MUFA	PUFA	
24h	*p=0.032			24h
3mo	*p=0.017		*p=0.008	3mo
6mo				6mo
12mo			*p=0.001	12mo
24mo			*p=0.019	24mo

	SFA	MUFA	PUFA	
24h				24h
3mo	*p=0.037	*p=0.005		3mo
6mo				6mo
12mo		*p=0.017		12mo
24mo		*p=0.001	*p<0.001	24mo



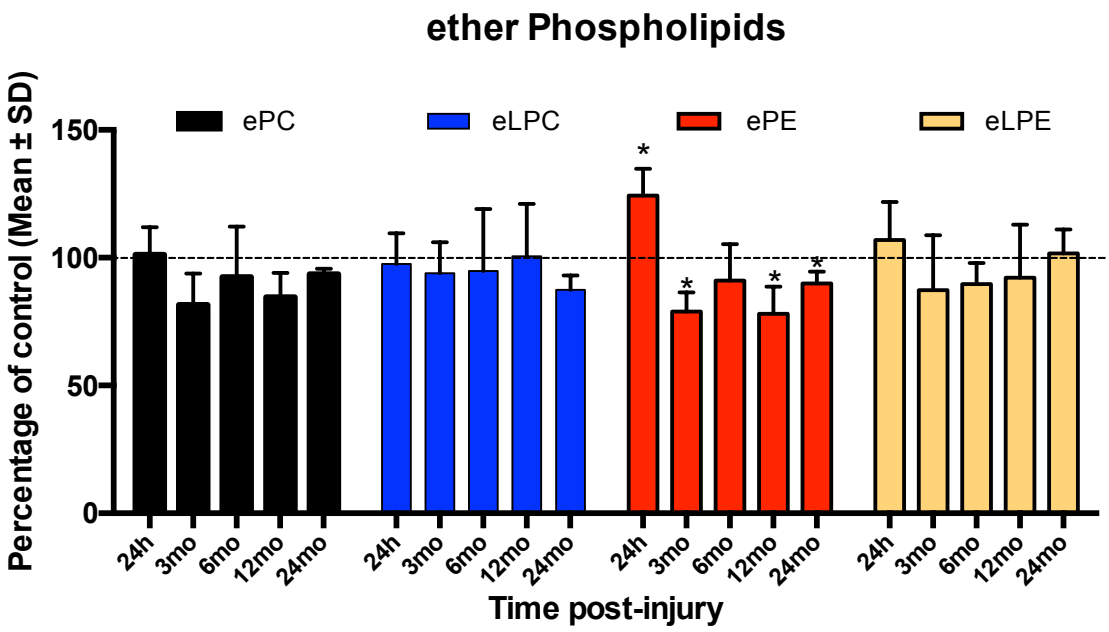
	SFA	MUFA	PUFA	
24h	N/A			24h
3mo	N/A		*p=0.033	3mo
6mo	N/A			6mo
12mo	N/A		*p=0.047	12mo
24mo	N/A		*p=0.006	24mo

Figure 4-5 Degree of unsaturation of PL classes in plasma of mTBI mice compared to control. Mean percentage of control ± SD. A) Significant changes of SFA ($p<0.01$), MUFA ($p<0.001$) and PUFA ($p<0.05$) containing PC species were observed over time. B) Within mTBI mice, SFA ($p<0.01$), MUFA ($p<0.001$) and PUFA ($p<0.001$) containing LPC species were significantly decreased at 3mo and 24mo post injury. C) Within mTBI mice, SFA containing PE species ($p<0.01$) were significantly increased 24h after injury and decreased by 3 mo, whereas MUFA-containing PE species showed no change after mTBI. PUFA-

containing PE species ($p<0.01$) were significantly decreased at 3mo, 12mo and 24mo. D) SFA ($p<0.01$), MUFA ($p<0.01$) and PUFA ($p<0.01$) containing LPE species showed significant changes in mTBI mice compared to control animals. E) Within mTBI mice, MUFA-containing PI species showed no significant difference ($p>0.05$). PUFA-containing PI species ($p<0.05$) were significantly decreased at 3mo, 12mo and 24mo, *$p<0.05$; MLM regression with post hoc analysis. The table gives p values for the timepoints

Examination of ether lipids in plasma of mTBI injured mice compared to control animals

Since ether PC and ether PE are dependent upon peroxisomes for their synthesis, we grouped these lipids separately. Figure 4-6 shows that ePE levels were significantly different in injured mice compared to controls ($p<0.05$). Ether PE species were increased 24h post injury and then decreased afterwards, significantly at 3, 12 and 24 months. Ether containing PLs in other classes were not altered after post-hoc correction.



	ePC	eLPC	ePE	eLPE	
24h			*p=0.029		**24h**
3mo			*p=0.035		**3mo**
6mo					**6mo**
12mo			*p=0.026		**12mo**
24mo			*p=0.045		**24mo**

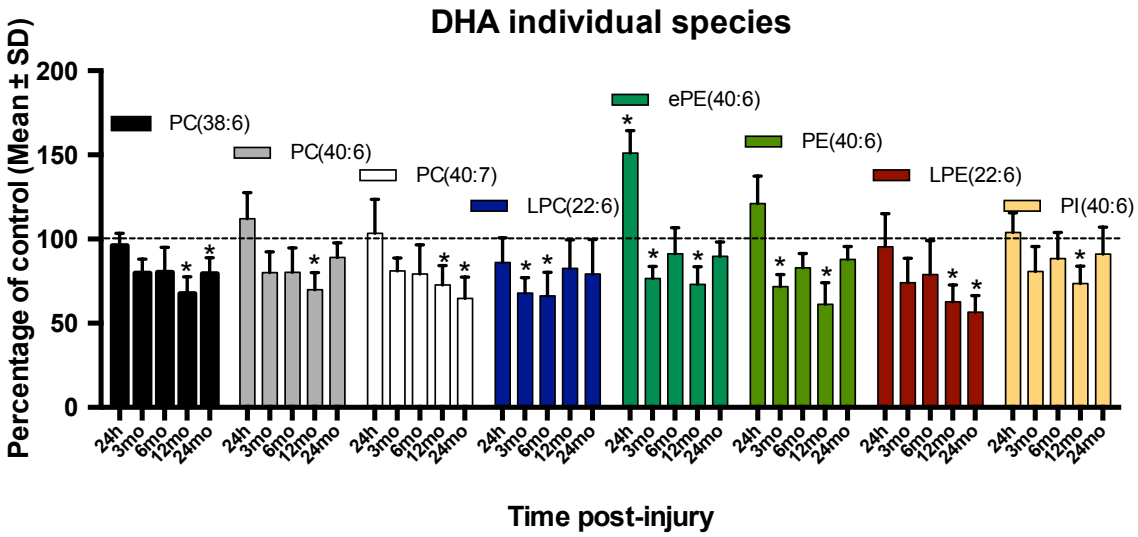
Figure 4-6 Differences in total ePC, eLPE, ePE and eLPE in plasma of mTBI mice. Mean values expressed as a percentage of control ± SD. Levels of ePC, eLPC and eLPE were not significantly different in mTBI mice compared to control. Ether PE species were increased 24h post injury before showing a decrease at 3mo and 12mo ($p<0.05$), p*<0.05; MLM regression with *post hoc* analysis. The table gives p values for the timepoints, where changes between mTBI vs. control mTBI vs. control were significantly different.

Profiling of AA- and DHA-containing phospholipid species in mTBI mice

We examined AA- and DHA-containing species due to their potential role in inflammatory responses, which are shown to accompany the chronic pathology of mTBI. Figure 4-7 shows the individual DHA containing PL species in the plasma of mTBI mice compared to controls, whereas Figure 4-8 represents individual AA containing PLs. In general, DHA containing PL species (such as PC(38:6), PC(40:6), LPC(22:6), PE(40:6),

LPE(22:6) and PI (40:6); $p < 0.05$) were decreased at several chronic post-injury timepoints. However, ePE(40:6) was elevated at 24hr post-injury but significantly decreased at 3- and 12- months post-injury ($p < 0.05$).

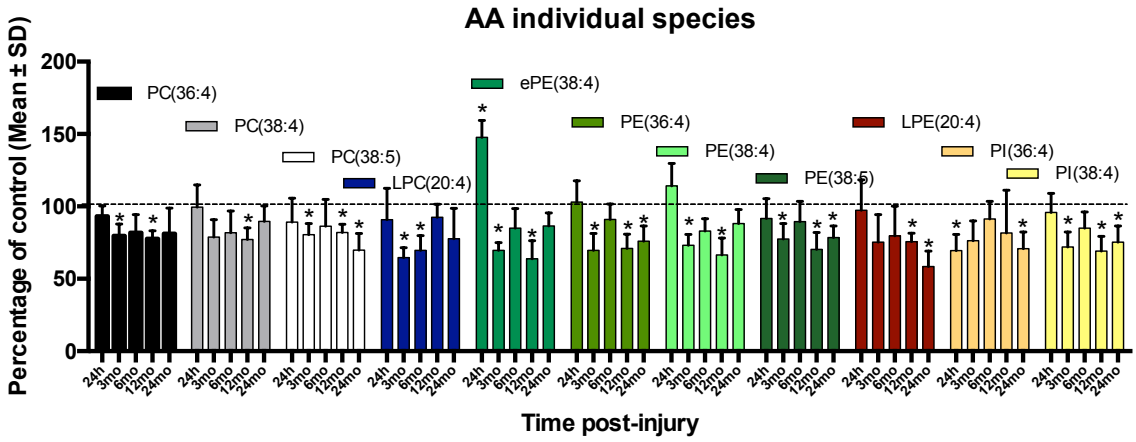
Similar to the results for DHA, in general, AA containing PL species were decreased at several chronic post-injury timepoints (such as PC(36:4), PC(38:4), PC(38:5), LPC(20:4), PE(36:4), PE(38:4), PE(38:5), LPE(20:4), PI(36:4) and PI(38:4); $p < 0.05$; Figure 4-8). As with DHA, an ePE species (ePE(38:4)) was increased at 24hr post injury and decreased at 3- and 12- months ($p<0.05$).



	PC(38:6)	PC(40:6)	PC(40:7)	LPC(22:6)	ePE(40:6)	PE(40:6)	LPE(22:6)	PI(40:6)	
24h					*p=0.001				**24h**
3mo				*p=0.034	* p=0.028	*p=0.01			**3mo**
6mo				*p=0.042					**6mo**
12mo	*p<0.001	*p=0.002	*p=0.008		*p=0.018	*p<0.001	*p=0.009	*p=0.019	**12mo**
24mo	*p=0.019		*p<0.001				*p<0.001		**24mo**

Figure 4-7 DHA containing phospholipid species in the plasma of mTBI mice compared to controls. Means expressed as a percentage of control ± SD. DHA containing PC species were significantly different in the mTBI group compared to controls ($p<0.05$). Within the mTBI group, DHA-containing PC(38:6) and PC(40:7) showed significant decreases at 12mo and 24mo post injury. PC(40:6) was only lower at 12mo. LPC(22:6) was significantly decreased at 3mo and 6mo post injury ($p<0.05$). DHA-containing PE species ($p<0.001$) revealed an increase at 24h for ePE(40:6) and, together with PE(40:6), showed a decrease at 3mo and 12mo post injury. For LPE(22:6) a decrease was observed at 12-, and

24month (p<0.01) for injured animals. PI(40:6) (p<0.05) showed significantly lower levels at 12mo. *p<0.05; MLM regression with *post hoc* analysis. The table gives p values for the timepoints where changes between mTBI vs. control were significantly different.



	PC(36:4)	PC(38:4)	PC(38:5)	LPC(20:4)	ePE(38:4)	PE(36:4)	PE(38:4)	PE(38:5)	LPE(20:4)	PI(36:4)	PI(38:4)	
24h					*p=0.003					*p<0.001		**24h**
3mo	*p=0.028		*p=0.035	*p=0.008	*p=0.007	*p=0.003	*p=0.007	*p=0.036			*p=0.031	**3mo**
6mo				*p=0.045								**6mo**
12mo	*p=0.003	*p=0.018	*p=0.039		*p=0.011	*p=0.017	*p=0.001	*p=0.012	*p=0.05		*p=0.034	**12mo**
24mo			*p=0.001			*p=0.004		*p=0.01	*p<0.001	*p=0.001	*p=0.003	**24mo**

Figure 4-8 AA containing phospholipid species in plasma of mTBI mice compared to control. Mean expressed as a percentage of control ± SD. Within mTBI mice, AA containing PC species were significantly different in mTBI mice compared to controls (p<0.05). AA-containing PC(36:4) was decreased at 3mo and 12mo post injury, whereas PC(38:4) showed significant changes only at 12mo. PC(38:5) was reduced at 3mo,12mo and 24mo. For LPC(22:4) we observed a significant decrease in mTBI animals (p<0.01) 3mo and 6mo post injury. AA-containing PE species ePE(38:4) revealed an increase at 24h and a decrease at 3mo and 12mo. PE(36:4) and PE(38:5) were both lower at 3mo and 12mo post injury. PE(38:4) was significantly decreased at 3mo, 12mo and 24mo. LPE(20:4) (p<0.001) showed lower levels at 12mo and 24mo. AA-containing PI species revealed lower levels of PI(36:4) in injured mice at 24h and 24mo, (p<0.05), whereas PI(38:4) was significantly reduced at 3mo, 12mo and 24mo post injury, *p<0.05; MLM regression with *post hoc* analysis. The table gives p values for the timepoints, where changes between mTBI vs. control mTBI vs. control were significantly different.

Profiling of individual molecular species of PC, LPC, SM, PE, LPE and PI in the plasma of mTBI mice.

Appendix Tables 2-7 (Appendix, Chapter 4; Section 2) show the concentrations of

individual phospholipid molecular species in plasma, grouped by class, that were identified by PCA to be significantly associated with injury at different time points.

For PC, multivariate PCA of the plasma data shows that component 1 explained 81% of variance in the dataset and was the strongest predictor of mTBI ($p < 0.001$) of individual PC species at time points 24hrs, 6 months (PC(32:0) only) and 24 months post-injury. Components 2 and 3 were associated with the 3 and 12 month timepoints. PC(42:6) was only modulated at the acute timepoint, whereas PC(34:1), PC(38:6), PC(40:5) and PC(40:7) started to be significantly lower at the most chronic timepoints of 12 and 24 months post injury. Appendix Table 2 shows individual PC species that were significant for different time points post injury.

For LPC, component 1 of the PCA showed significance for mTBI, which explained 70% of the total variance in the dataset ($p < 0.05$) and was associated with 24hr, 3, 6, and 24 months post injury. For the acute timepoint only LPC(16:1) was significantly modulated in mTBI mice and LPC (o-16:1) showed a 46% decrease by 24 months post injury. Appendix Table 3 shows all individual LPC species that were identified as significant by PCA in component 1 for different timepoints post injury.

For PE, multivariate PCA of the plasma data showed that component 1 explained 68% of the variance in the dataset and was a strong predictor for injury ($p < 0.001$) at timepoints 24hrs, 3-, 12-, and 24 months. For the acute timepoint several species (e.g. ePE(38:5), ePE(38:6) and PE(38:0)) showed an increase of between 66-72%. The same species were observed to be decreased at chronic stages. Appendix Table 4 shows individual PE species that were significant for different timepoints post-injury.

For LPE, component 1 of the PCA, explained that 52% of the total variance in the dataset ($p < 0.05$) was significantly associated with mTBI for all chronic timepoints. No individual LPE species was different from control at 24hrs post-injury. Appendix Table 5 shows individual LPE species that were specific to PCA component 1.

For PI, component 1 explained 66% ($p < 0.05$) of the total variance and was a predictor for mTBI for all timepoints. Appendix Table 6 shows individual PI species in plasma with significant changes for the different timepoints post-injury.

For SM, component 1 explained 59% of the total variance ($p < 0.05$) and was significantly associated with mTBI at 6-and 24 months post-injury. No significance was observed at the acute timepoint. Moreover, SM species SM(d18:1; 22:1) and SM(24:0) were only modulated at 24 months post injury. Appendix Table 7 shows individual significant SM species that were specifically significant with PCA component 1.

Lipid peroxidation at 24hrs and 3months post injury

The PL analysis revealed that there were no differences between control and mTBI mice at 24hrs post injury, whereas we observed a significant decrease for many PUFA-containing PL species at 3 months. Thus we investigated lipid peroxidation at both timepoints by measuring MDA production (Figure 4-9 A-B). No difference was observed for mTBI and control mice at 24hrs post injury ($p > 0.05$). At 3 months, we observed a significant increase for MDA in mTBI mice compared to controls with a fold change of 1.77 ($p = 0.003$).

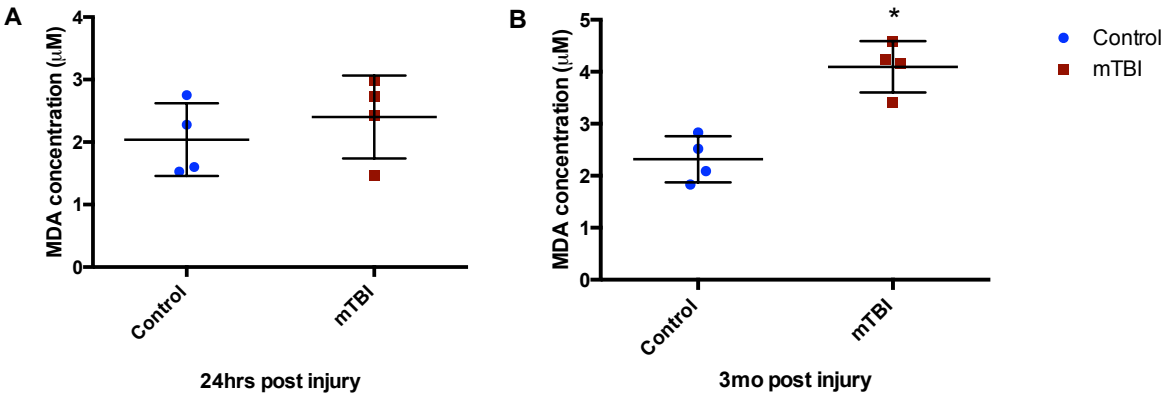


Figure 4-9 MDA concentrations in plasma of mTBI mice compared to control at 24hrs and 3 months post injury. (A) No differences were observed in peroxidation levels between control and mTBI mice at 24hrs post injury ($p>0.05$; control: 2.04 ± 0.58µM; mTBI: 2.40 ± 0.67µM in mean ± SEM. **(B)** MDA concentration was significantly increased in mTBI mice (n=4) compared to control (n=4) at the 3 months timepoint ($p=0.003$; control: 2.32 ± 0.44µM; mTBI: 4.10 ± 0.49µM).

Change of LRG1 levels over time

The LRG1 ELISA showed no differences 24hrs after injury between mTBI mice and controls (Figure 4-10). At the 3 month timepoint marginally higher levels of LRG1 were observed in mTBI animals (Prob>Chi square $p<0.01$), whereas at 6 months changes between the two groups become more significant with injured animals showing increased levels of LRG1 (Prob>Chi square $p<0.009$). Although LRG1 levels did not decrease in mTBI mice at the 12 and 24 months timepoint, the concentration of this protein rose in control animals with age, and thus significance was lost at these the later time-points. Mean (and SD) concentration for LRG1 at each timepoint in plasma was observed as follows: 24hrs: mTBI 214.9 ± 45.1 µg/ml; Control 152.9 ± 30.3 µg/ml, 3 months: mTBI 310.9 ± 34.3 µg/ml; Control 189.2 ± 37.9 µg/ml, 6 months: mTBI 279.7 ± 29.3 µg/ml;

Control 172.8 ± 17.6 µg/ml, 12 months: mTBI 341.8 ± 55.7 µg/ml; Control 326.5 ± 32.3 µg/ml, 24 months: mTBI 272.8 ± 26.1 µg/ml; Control 298.8 ± 60.5 µg/ml.

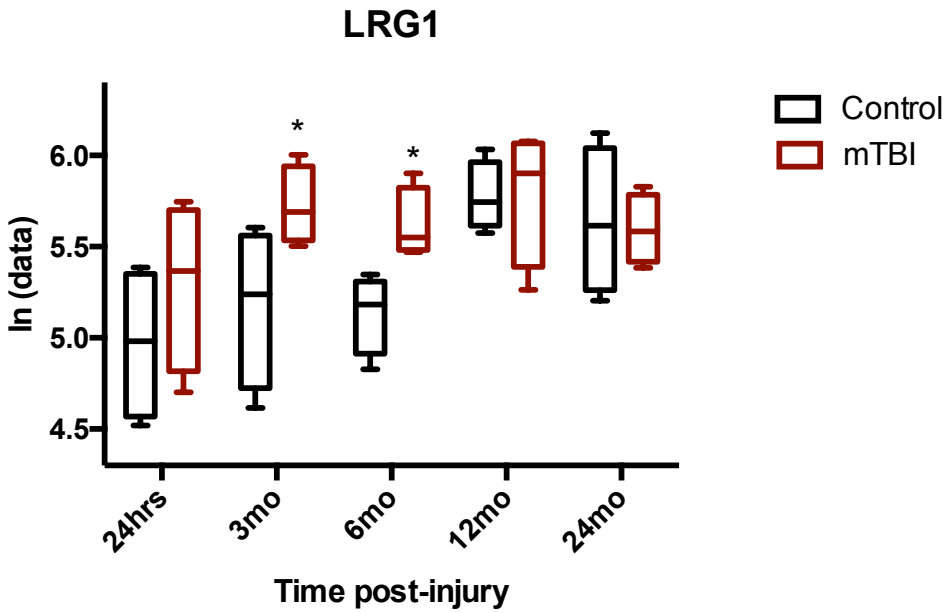


Figure 4-10 LRG1 concentrations in the plasma of mTBI mice compared to control at 24hrs, 3-, 6-, 12-, and 24 months post injury. No differences were observed in LRG1 levels between control and mTBI mice at 24hrs post injury ($p>0.05$). LRG1 concentration was significantly increased in mTBI mice (n=4) compared to control (n=4) at the 3 months ($p=0.01$) and 6months ($p=0.009$) timepoints. At 12 and 24 months no significant differences were observed, as levels in mTBI mice stay elevated, whilst LRG1 in control animals increased.

4.4 Discussion

The secondary damage that follows TBI includes inflammation, blood brain barrier dysfunction and axonal injury, which can persist over a long time period [340339]. Previous studies, including ours, have shown that PL levels are altered in subjects with a history of mTBI, and in a controlled cortical impact mouse model of relatively severe CCI induced TBI [348,349]. These findings suggest that PL may be important in the

pathophysiology of TBI and could in the future serve as biomarkers. They would also be useful for improving our diagnostic and prognostic capabilities for brain injury. Such biomarkers could be also used theragnostically, as surrogate markers for assessing the therapeutic response to potential TBI treatments. Given our previous findings suggesting PL biomarkers of TBI at chronic time points post-injury in a human population, we sought to determine if such changes were evident in our laboratory model of mTBI, which would support its relevance to the human population and lead to further translational studies.

We first examined PL profiles in control mice to determine if aging itself had an impact on plasma PL profiles. While LPC and LPE levels did not differ over time, PC, PE, PI and SM all showed a significant decrease at the 24month time point. At 24 months post injury (26.5 months of age) mice are nearing the end of their lifespan [350]. It is known that PL are the major components of membranes and play a role in maintaining the membrane integrity as well as in functional activity. Lipid metabolism and its turnover, especially oxidation of fatty acids, have been implicated in apoptosis, and aging causes an increase in molecular damage to the cell membranes and changes in the normal asymmetrical architecture of membranes [351–354]. Ovsepian et al. have shown that PC, PE and SM were decreased in the brains of 25-month-old rats, whereas LPC and PI increased. Moreover, a study by Kim et al. compared PC, LPC and SM levels in the sera of young (4 month) and old (21 month) mice and showed that lipid levels were decreased with age for PC and SM, as well as some of the LPC species [355]. Our findings confirm these results. Although the age-dependent decrease in LPC does not reach significance at 24 months in our study, a non-significant trend is visible. A decrease of PL at 24 months

post injury could be due to an increase in oxidation. Yet, further studies are needed in order to understand the biological pathway underlying these changes. When evaluating the aging effect in mTBI mice we observed the same decreases as in control mice. However, additionally, we observed a significant decrease in LPC at 24 months when compared to the 24hrs levels, as well as an earlier initiation (12 months) of the significant age-dependent decrease in PE. Because we used age-matched controls our subsequent analysis of mTBI versus control for each time point was not affected by the aging component. Yet, as seen by comparing control and mTBI mice with their 24 hrs counterparts, 24 months post injury, the TBI group showed lower PL levels, beyond that of "normal aging". Moreover, the fact that lower levels were also observed at 12 months could suggest an accelerated aging effect in the TBI animals. Although more investigation on this subject is necessary, a link between TBI and accelerated ageing has been suggested (reviewed by [356]). For example, people who had recovered from a minor TBI who were exposed experimentally to a stressful situation (induced mild hypoxia) had significantly impaired vigilance and memory abilities compared to healthy subjects [357].

In the case of mTBI, overall, total PL levels for all classes evaluated in this study did not show major differences at the acute 24 hrs post injury time point. We then focused our study on the impact of mTBI on plasma profiles over time post-injury. At 3-months post-injury, we observed a decrease in injured animals compared to their control counterparts in PC, LPC, PE, LPE and PI, but not for SM. Total PC, PE and PI also showed significantly lower levels at 12 months and all classes were decreased at 24 months post injury in the mTBI group compared to controls. The 6-month time point did not reach significance due to increased variance in control animals seen at this time point.

Higher variance was also observed for SM levels at time points prior to 24 months post injury.

Kay et al. were able to detect PL disturbances from the brain in the CSF in humans, showing that brain alterations can be detected in peripheral biofluids [310]. Moreover, Abdullah et al. investigated PL profiles in different brain regions, as well as plasma at 3 months post injury in a CCI model showing that even at chronic stages post injury, PL changes persist [349]. We also conducted a clinical study involving active duty military with a history of mTBI and we detected changes in plasma lipids at this chronic stage (Chapter 3). It is important to note that clinical evidence from our military study confirms the findings of this study. Total PL levels were decreased in soldiers with mTBI compared to controls for all PL classes [348].

Overall, lipid levels showed no difference in mTBI mice compared to control at 24hrs post injury but changed over the next 3 months as indicated by the decrease in mTBI versus controls at the 3 months timepoint. We then observed a possible recovery phase around the 6 months timepoint, where PL levels returned to those of control animals. Finally, lipid levels dropped again at the chronic 12- and 24 months timepoints post injury.

It is known that saturated and monounsaturated fatty acids can be synthesized *de novo* within the brain, however polyunsaturated fatty acids are mainly supplied from the periphery [318]. In this study, we observed that saturation status, specifically levels of unsaturation, was different in injured compared to control mice.
Overall SFAs, MUFAs and PUFAs were decreased for PC, LPC and LPE at different time points. In our clinical study we also showed differences in PL degree of

unsaturation and its decrease at the chronic stage. It is interesting to observe that SFAs, MUFAs and PUFAs are differently regulated within different PL classes. It is also interesting to mention that in mice, MUFAs of PE and PI did not reach significance at any time point. However, in order to better understand the molecular mechanisms of PL involvement following TBI and their unsaturation status, it will be necessary to investigate enzymes that are involved in their BBB transport, desaturation of SFAs and/or elongation of MUFAs to PUFAs. This study was not intended to investigate specific aspects of TBI pathogenesis as it related to lipid metabolism, but rather to develop potential biomarker profiles. Nonetheless, we were interested in whether or not lipid peroxidation was a factor in our findings and we found total lipid peroxidation to be elevated in mTBI compared to control at 3 months post injury chronic timepoint when PUFA levels were significantly reduced. As PUFAs, owing to the presence of multiple double bonds, are susceptible to oxidative degradation these findings of higher levels of lipid peroxidation are consistent with the observed decreases in PUFA levels at this timepoint. However an assay with the sensitivity to detect oxidized PL by MS needs to be employed to draw further mechanistic conclusions.

We analyzed ether-containing PLs and characterized their response to injury. Changes in ether PLs (LPC o-16:0 and LPC o-18:0), which are precursors to inflammation promoting platelet-activating factors (PAFs) were found to be significant. Although eLPC were not significantly modulated by mTBI, LPC (o-16:0) was significantly lower at 24 months post injury. Furthermore, for other ether lipids, such as ePC and eLPE, no significant differences were observed. A majority of ether PE species are plasmalogens [297]. We observed increased ePE 24 hrs post injury before it decreased at

3 months and again at 24 months. This correlates with our clinical studies, where we only observed lower levels of ePE in TBI subjects, all of whom were recruited at chronic time points after injury. As ePEs are enriched for DHA and AA, serving as reservoirs for their bioactive lipid metabolites[297], a decrease in these lipids could mean increased synthesis of eicosanoids and docosanoids that modulate inflammation following TBI. However, specific monitoring of bioactive lipid metabolites will be necessary to draw further conclusions in this regard.

It is known that bioactive metabolites of DHA and AA, the most abundant ω-3 and ω-6 PUFAs in the brain, contribute to the immune and inflammatory balance within the CNS and the periphery. DHA is a precursor of the anti-inflammatory mediators, resolvins, and neuroprotectins, whereas AA is a precursor of pro-inflammatory lipid mediators, such as prostaglandins and leukotrienes [319,320]. Therefore we further investigated DHA and AA containing lipid species. We only observed a significant reduction in the AA:DHA ratio in TBI mice for the lipid class PE, at 24 hrs post injury (data not shown). This correlates with our clinical data, where we observed reduction of AA to DHA containing molecular species only in PE for mTBI subjects compared to controls. Although ratios didn't change for other lipid classes in mice, we observed changes in specific DHA-containing and AA-containing PL species in mTBI versus control mice. For the acute 24 hrs time point, we observed a significant increase in AA containing ePE(38:4) as well as in DHA-containing ePE (40:6). Although no other AA or DHA containing species reached significance at 24 hrs, PC(40:6), PE(40:6), as well as PE(38:4) showed a trend of higher levels. This correlates with a clinical study by Yang et al., who showed that AA was enhanced in serum metabolites in TBI subjects shortly after

injury [358]. Apart from elevation at the 24 hrs timepoint, individual DHA and AA containing species were significantly decreased at several chronic stages in mTBI mice compared to control animals. As the brain particularly needs DHA, there may be an increased need for DHA containing lipids after injury for repair and other related functions [322–324]. Therefore the acutely lowered AA:DHA ratio could reflect the early DHA increase in response to injury, and subsequent decrease owing to their increased utilization. Tracking the changes in plasma AA and DHA in PL could potentially serve as a surrogate marker once it is confirmed that AA and DHA changes in plasma reflect brain changes. Furthermore, truncated products, such as prostaglandins and resolvins of AA and DHA, respectively, should also be evaluated. Moreover, we hope to replicate this study in mTBI mice of other strains and genetic backgrounds, which will help further verify our work. Our focus in recent years has been on mild TBI; therefore this study does not include severe head injury paradigms, though for future study this would definitely be an interesting area of investigation and comparison.

Furthermore, the observed injured animals showed neuroinflammation and white matter loss, which peaked at 6 months and remained static at the 12 months timepoint compared to controls [342]. Although it is not possible at this point to relate plasma PL chances with the observed persistent neuroinflammation, the ongoing dyregulation of PL could be related to the pathology of this injury model. However, in order to better understand this connection, PL should be measured in the same brain regions (e.g hippocampus), in which neuroinflammation was detected. As this mild TBI model does not show gross structural changes more severe injury or repetitive mild TBI models could

be used to investigate if resulting structural changes, as well as a stronger neuronflammatory response relate to PL in brain as well as plasma.

Finally, our measurements of LRG1 concentration in our mouse model revealed interesting findings. No differences were observed at the acute 24hrs timepoint. However, significant elevations of LRG1 in mTBI mice were seen at the 3 month with significance increasing at 6 months. At later timepoints (12 and 24 months) the LRG1 concentration in the plasma of control animals was raised as well, resulting in a loss of significance between the two comparison groups. This is of interest, as it appears that aging has an effect on LRG1 levels. In our proteomic study in human samples (Chapter 2), we observed a significant increase in LRG1 concentration compared to controls in the TBI and TBI+PTSD group as well, matching the mice 3 month and 6 month timepoint.
As previously described, LRG1 was proposed as a modulator of angiogenesis, acting via TGFβ1 endothelial cell signaling [359]. Angiogenesis has been proposed to play a role in mediating functional recovery after TBI, as tested neurorestorative agents have been shown to improve functional outcome after TBI and increase angiogenesis (reviewed by [360]. Furthermore, acute increases in $CD34^+$ endothelial progenitor cells (EPC), an inducer of angiogenesis, have been observed after TBI in peripheral blood and brain tissue of rats [361]. Another study investigated angiogenesis via vascular endothelial growth factor (VEGF) and VEGF receptor 2 expression and showed increases up to 48hrs post injury [362]. Although these major regulators of angiogenesis are believed to resolve within days after injury, other mechanisms could be ongoing. As LRG1 is suggested to act via the ALK1-Smad1/5/8 pathway, increased levels at more chronic timepoints could indicate a pro-angiogenic transcriptional response. However, further studies are needed to

understand the mechanism behind LRG1 in TBI not at acute but chronic timepoints. Finally, TGFβ1 signaling is induced with age [363,364]. Perhaps this could explain why LRG1 is increased in controls as well at later timepoints. However, further investigation is needed to understand the potential role of LRG1 in TBI. Finally, known potential acute biomarkers, such as S100β, GFAP, Tau and UCH-L1 could be determined in this cohort the 24hrs, as well as sub-acute 3month timepoint and be used as a "positive control".

4.5 Conclusion

We were able to confirm that our human findings of altered plasma PL profiles after TBI are also seen in our mouse model, validating it as a platform for future translational studies. This will include investigating the underlying molecular pathways that are associated with perturbed plasma PL profiles and LRG1increases in TBI. Moreover, this mTBI model has previously shown pathological and behavioral features, over time points with which these changes correlate, which are comparable to those observed in human subjects. Additional investigation of lipid metabolizing enzymes, lipid transport and oxidation studies will build our understanding of the biological mechanisms behind the PL observations. Furthermore, in our clinical study we observed a strong influence of APOE genotype on plasma lipid levels. The study of mTBI in mice genetically modified at the APOE locus could bring further understanding of the effects of mTBI, to not only enable the development of biomarkers but also the discovery of molecular targets for personalized medicine.

Chapter 5 Phospholipid profiling of plasma from GW veterans and rodent models to identify potential biomarkers of Gulf War Illness

5.0 Summary

In this chapter we investigated another debilitating military condition, namely Gulf War Illness (GWI). GWI is characterized through chronic heterogenic symptoms, including pain, fatigue and cognitive problems. As a consequence this illness remains difficult to diagnose. Rodent models have been shown to exhibit symptomatic feature of GWI through exposure to GW-agents (pyridostigmine bromide, permethrin and DEET) and/or stress paradigms and preclinical analysis has shown activation of microglia and astroglia as a pathological hallmark. Although much has been learned in recent years from those models as well as independent clinical studies, translational studies between animals and humans have been lacking. Thus, in this study we aimed to identify biomarkers, which are translational between plasma of rodent models of GWI and human patients. We observed increases of multiple phospholipid (PL) species across all studied cohorts. Furthermore, our data suggest dysfunction within ether- and DHA- and AA-containing PL species, supporting the proposed role for immune and inflammatory balance with veterans with GWI. Overall, we show that peripheral lipid disturbances can be found not only in humans but as well in the studied animal models. This emphasizes the value of further study in these models, as well as the use of lipidomics as a potential platform for further biomarker discovery for objective diagnosis.

5.1 Introduction

Gulf War Illness (GWI) is a chronic multisymptom illness, which affects approximately one fourth of the 700,000 US veterans, who were deployed to the 1990-91 Persian Gulf War (GW) [197,198,200,201,233]. Veterans with GWI experience chronic health symptoms such as fatigue, muscle and joint pain, and gastrointestinal problems [221]. Among the central nervous system (CNS) based symptoms, memory problems are among the most commonly reported complaints [198,200,205,221,365]. The multiplicity and heterogeneity of symptoms observed in GW veterans is unique to the 1990–91 deployment, with no identical illness being reported in any other military campaign, indicating that GWI etiology cannot solely be attributed to combat-related stress [198,205,210,366–368]. Twenty-five years later, GW veterans are still coping with this chronic illness, which remains difficult to diagnose due to a lack of objective diagnostic tests. Currently, clinical diagnosis is most commonly made by self-report of health symptoms using the Fukuda CDC criteria or the Kansas GWI criteria [198,200,233]. Consequently, many GW veterans report symptoms consistent with GWI but have not received a formal diagnosis of their condition. Blood based biomarkers of GWI are needed to assist clinicians with providing an objective diagnosis of GWI and for developing targeted treatment strategies.

After performing a comprehensive review of clinical and animal research conducted to identify the causes of GWI, the Research Advisory Committee (RAC) on GW Veterans' Illness concluded that the key contributors to GWI etiology included combined war-time exposure of the prophylactic anti-nerve gas pill pyridostigmine

bromide (PB) and pesticides which were also used prophylactically to protect against insect-borne diseases [197,221,233]. The many pesticides used during the GW included irreversible and reversible acetylcholinesterase inhibitors (AChEi) including organophosphate (OP) and carbamate pesticides [197,221]. Other commonly used pesticides included pyrethroids, such as permethrin (PER), the insect repellant N, N-diethyl-m-toluamide (DEET) and organophosphates such as diisopropyl fluorophosphate (DFP) [197,210,366,367,369–373]. OP exposures from low-level sarin nerve agent exposure also has occurred in many GW veterans and has been modeled in animal studies with the sarin surrogate DFP. Results of these studies showed a chronic neuroinflammatory phenotype as a result of this sarin-surrogate exposure, particularly when in conjunction with a physical stressor [374].

The complex clinical presentation of GWI suggests that various biological and metabolic processes might be altered in GW veterans. For example, impaired immune responses have been observed in veterans with GWI [221]. Veterans with GWI showed altered expression in pro-and anti-inflammatory cytokines on their peripheral immune cells, and immune system irregularities were especially pronounced in GWI patients compared to controls during exercise challenge, such as increased IL-6, IL-10, and TNF-α levels, as well as a Th1/Th17 immune polarization [223–227].

Phospholipids (PL) are critical components of most cellular membranes, including mitochondrial membranes, and their metabolism generates bioactive lipids that can modulate inflammatory pathways [290]. In particular, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) serve as a reservoir for ω-3 docosahexaenoic acid (DHA) and ω-6 arachidonic acid (AA), metabolism of which generates bioactive lipid, which can

alter the immune/inflammatory responses in both the central and the peripheral nervous system. For instance, DHA is a precursor of the anti-inflammatory mediators, resolvins and neuroprotectins, whereas AA is a precursor of pro-inflammatory lipid mediators, such as prostaglandins and leukotrienes [319,320]. Preclinical studies have shown that exposure to GW agents can affect the brain phosphocholine levels [229], which is a metabolite of PC required for the endogenous synthesis of acetylcholine (ACh) [375]. In addition, mitochondrial dysfunction is thought to underlie the clinical features of GWI [230,231]. Furthermore, mitochondria also contain PL, of which PC and PE make up approximately 75% of PL and phosphatidylinositol (PI) constitute 10% [376].

Our group previously established several mouse models using exposures to combinations of these GW agents in order to investigate the complex underlying GWI pathology, which presumably involves multiple physiological and biochemical mechanisms. We previously showed that PB+PER co-administration in a CD1 mouse model daily for 10 consecutive days, led to observed anxiety 30 days post exposure and delayed cognitive impairment 5 months post exposure [367]. Additionally, at 5 months post exposure, astrogliosis was observed in exposed mice. Proteomic analyses revealed alteration of multiple biological systems, such as endocrine function, immune and inflammatory pathways, as well as disturbances in lipid metabolism [367]. For example, the fatty acid-binding protein 3 (FABP3) was reduced in the brain of exposed animals. This protein plays an important role in the uptake of AA in the brain [377]. We subsequently translated the PB+PER exposure model to the more commonly used C57BL6/J strain of mice, again showing cognitive deficits associated with increased astrogliosis, as well as reduction of synaptophysin staining in the hippocampi and cerebral cortices at 5 months

post exposure [378]. Since the neurobehavioral features we observed in our mouse model correlate with symptoms that are relevant to the clinical presentation of GWI, it is possible that identified pathologies in these animals are also present in human GWI patients [200,379]. We have also reported elevated ether and diacyl PC species in the brains of these mice [229].

The insect repellant DEET has also been named one of the key contributors to GWI, and it has been suggested that stress modulates GWI symptoms in humans [201,379]. Addressing these additional exposures, Shetty and colleagues developed and characterized a rat model of GWI using a PB+PER+DEET exposure paradigm, along with 5 min of restraint stress to mimic war related stress [210,380,381]. Their rat GWI model showed memory and mood dysfunction evident by deficits in spatial memory and increased depressive behavior [380]. They also observed some loss of glutamatergic neurons, activated microglia, hypertrophy of astrocytes, reduced neurogenesis [380] and loss of certain subpopulations of gamma-amino butyric acid-ergic (GABA-ergic) interneurons in the hippocampus [382].

These mouse and rat studies have been critical for advancing our understanding of the different facets of GWI pathology and for characterizing the underlying biological features. However, translational studies between different animal models and human patients are currently lacking. Furthermore, there remains a need for identifying novel biomarkers, which can assist with diagnosing GWI and may also help link preclinical and clinical studies aimed at developing therapies. Taking into account the role of lipid disturbances detected in preclinical rodent models of GWI and the need for translational validation studies, we performed lipidomic analysis in the plasma of the two different

rodent models described above, which we then compared to results from plasma analysis of a cohort of GW veterans with and without GWI.

5.2 Materials & Methods

Animals

GWI mouse model:

All mouse experiments were conducted at the Roskamp Institute in Sarasota, Florida and were approved by the Roskamp Institute's Institutional Animal Care and Use Committee and conducted in accordance with the Office of Laboratory Animal Welfare and the Association for the Assessment and Accreditation of Laboratory Animal Care. Mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and each mouse was individually housed in a controlled environment (regulated 14-h day/10-h night cycle) and maintained on a standard diet. The exposure paradigm has been previously described [378,383]. Male C57BL6/J mice (12 weeks of age) were co-administered with either a 50µl total volume of GW agents to a final dose of 0.7 mg/kg of PB (Fisher Scientific (Hanover Park, IL)) and 200 mg/kg of PER (Sigma Aldrich (St. Louis, MO)) in 100% dimethyl sulfoxide (DMSO) [exposed mice; n = 4], or a 50µl volume of vehicle (100% DMSO) [sham mice; n = 4] via intraperitoneal injection (IP) injection daily, for 10 days. Six months post exposure, mice were euthanized, and plasma was collected. Mice were exsanguinated via cardiac puncture using an 18 gauge wide-bore needle to prevent hemolysis of red blood cells (RBC) during blood collection. Blood samples were collected into a 1.5 ml Eppendorf tube containing 10 units of heparin and a protease

inhibitor cocktail (Roche, NJ) to a final concentration of 1x. Samples were immediately centrifuged at 3000 x g for 5 min and the plasma was transferred to a new 1.5 ml Eppendorf tube and snap frozen in liquid nitrogen. Plasma samples were stored at -80C° until further biochemical studies.

GWI rat model:

All rat studies were conducted at the Central Texas Veterans Healthcare System in Temple, TX. Specifically, Sprague-Dawley rats were exposed to PB, DEET, PER and Stress using previously described procedures developed by Shetty et al. [380,381]. Experiments were in compliance with the National Institutes of Health guidelines for care and use of animals and in accordance with the animal protocol approved by the animal care and use committee of the Central Texas Veterans Health Care System, Temple TX. Three-month-old male rats were obtained from Harlan (Indianapolis, IN) and housed for 2 weeks before being exposed to GW agents. The exposure paradigm included PB (1.3 mg/kg; Sigma, St. Louis, MO), PER (200 µl, 0.13 mg/kg in 70% alcohol; Chem. Service Inc., West Chester, PA), DEET (200 µl containing 40mg/kg in 70% alcohol; Chem. Service Inc., West Chester, PA), as well as 5 min of daily restraint stress, via a rat restrainer for 4 weeks (exposed rats n=5). PB was administered through oral gavage, whereas PER and DEET solutions were applied to shaved skin areas located on the back of neck. Non-exposed sham rats, were naïve controls (n=6). Plasma samples were collected 6 months post-exposure to GW agents and stress, and were sent to the Roskamp Institute investigators on dry ice via express delivery.

Human subjects

Plasma samples from veterans, deployed to the 1990-1991 GW, were provided by the Boston University and the Nova Southeastern University investigators from an established biorepository of GW veterans, who agreed to share their blood samples from prior studies. This includes the Boston Gulf War Illness Consortium (GWIC) and the Dynamic Modeling of GWI study from Nova Southeastern University and the CDMRP funded study to the South Florida Veterans Affairs Foundation for Research and Education, Inc. The GWI biorepository is approved by the IRBs from Boston University, Nova Southeastern University and the Miami VAMC. Samples from the GWI biorepository were all collected from the Boston and Miami areas using the same standard operating procedures for phlebotomy, plasma separation and aliquoting. All samples were stored at -80^{o}C and were not previously thawed and refrozen. GW veteran participants were consented into their respective studies using ICH GCP guidelines. The Kansas GWI criteria [200] were used to determine cases of GWI and controls. The Kansas GWI criteria require that GW veterans endorse symptoms in at least 3 of 6 symptom domains (fatigue/sleep problems, pain, neurological/cognitive/mood symptoms, respiratory complaints, gastrointestinal problems or skin symptoms). Controls were deployed veterans from the 1991 GW who did not meet the Kansas GWI criteria. Subjects also completed demographics and health symptom questionnaires including the Pittsburgh Sleep Quality Index (PSQI), Visual Analog Scale (VAS) for pain, Multi-dimensional Fatigue Inventory (MFI-20) questionnaire, MOS Short Form 36-veteran version (SF-36V), and Profile of Mood States (POMS). Questionnaires can be found in the appendix, chapter 5; section 1. Study participants were excluded if they reported

being diagnosed with another medical condition that could explain the above mentioned symptoms according to the Kansas GWI case definition exclusions, including veterans with a history of prior central nervous system or major psychiatric disorders that may affect cognitive function (e.g., epilepsy, stroke, brain tumor, multiple sclerosis, Parkinson's Disease, Alzheimer's disease, schizophrenia). Control veterans (n=11) and veterans with GWI (n=22) were matched for age, gender and ethnicity. Due to low numbers in the different ethnic groups, we dichotomized the ethnicity into Caucasian (n=17) vs. non-Caucasian (n=16) in order to study ethnicity effect on PL levels in the entire cohort population independently of diagnosis. The non-Caucasian group included African American (n=8), Hispanic (n=4), Asians (n=2) and others (n=2). Baseline demographics can be found in Table 1.

Lipidomic and Statistical Analyses were performed as previously described in Chapter 3.

5.3 Results

Our aim was to first establish a plasma PL profile that could distinguish veterans with GWI from deployed controls. We examined changes in total PL levels, (PC, PE, PI, LPC and LPE), as well as the degree of unsaturation of PL class, ether content and ω-3 and ω-6 fatty acid (FA) composition. We then investigated the association of individual PL molecular species with the diagnosis of GWI. Finally, we examined these species in rodent models of GWI in order to be able to validate these preclinical models. Our hypothesis is that if blood changes in the mouse models correlate with GWI-dependent plasma profiles in humans, then we may be able to extrapolate from plasma to brain, mouse to human for potentially pathogenic mechanism. Specifically, although ambitious, we suggest correlation of our findings in human plasma to mouse plasma, and correlation of these plasma changes with changes we observe in the mouse brain; and we hypothesize that such GW-agent dependent changes in the mouse brain, correlating with mouse exposure/human GWI -dependent plasma changes, may thus reflect changes in the human GWI patient brain.

Comparison of total phospholipid classes in GWI patients and GW deployed controls

Our case control cohort included a total of 33 veterans who were deployed to the GW region during 1990-1991; 11 were classified as controls and 22 veterans were

diagnosed with GWI. In comparing baseline characteristics, control and GWI veterans were similar for gender, ethnicity and age (see Table 5-1).

	Control	GWI
N total	11	22
Age (Mean±Stdev)	48.5±7.7	48.4±6.3
Male (%)	9 (81.9%)	17 (77.3%)
Ethnicity		
Caucasian	5	12
African American	3	5
Hispanic	2	2
Asian	0	2
Other	1	1

Table 5-1 Baseline demographics of the Gulf War Veterans cohort.

In order to determine if gender, age and ethnicity could potentially be confounding factors on the relationship between GWI and PL, we examined their influence on PL levels in the entire cohorts (combined controls and GWI). A gender effect was only observed for total PE ($p=0.004$) levels. There was no significant influence of ethnicity for any of the PL classes. There was a significant correlation of age with LPC ($r=0.49$; $p<0.001$) and PI ($r=0.27$; $p=0.005$).

We investigated changes in total PL levels for GWI cases compared to controls, presented as a percentage of control and shown in Figure 5-1. For all subsequent analyses, we used gender as a covariate for all analyses pertaining to PE and age as a covariate for PI and LPC in order adjust for their potential confounding effects on relevant PL class. There were no significant differences for total PC, PE, LPE and PI

between the two comparison groups ($p>0.05$). However, for total LPC post-hoc analysis revealed a significant increase of 15% for GWI patients versus controls ($p=0.020$).

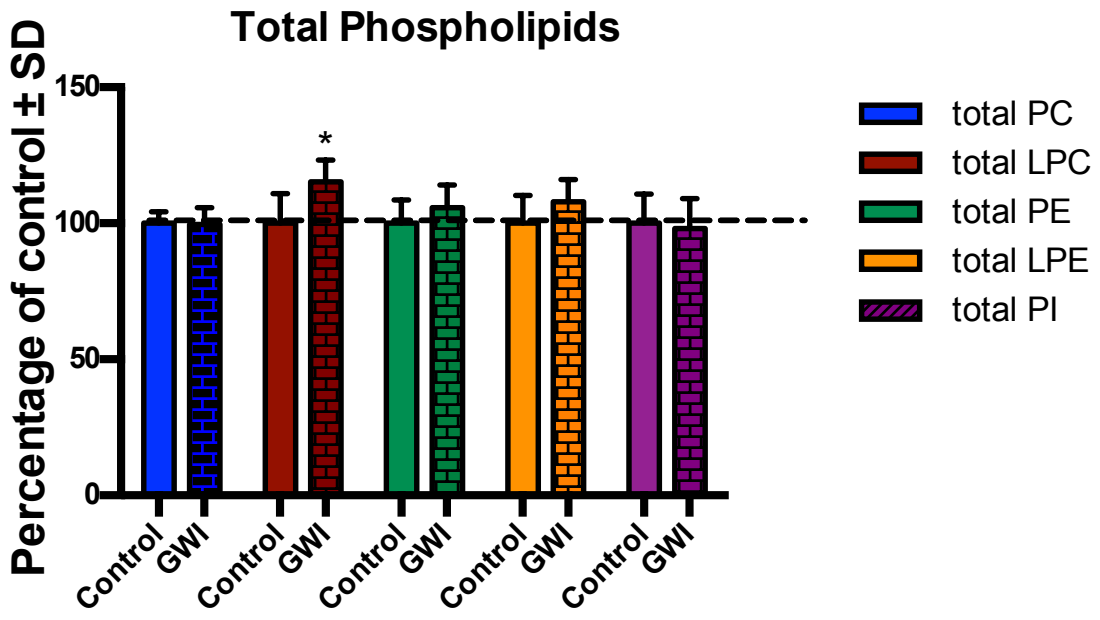


Figure 5-1 Changes in total plasma phospholipid levels in GWI subjects and controls, represented by the mean ± SD as a percentage of control. Individual molecular species for each PL class identified by LC/MS were summed to calculate total PL levels within each class. * $p < 0.05$; MLM regression with *post hoc* analysis. Total LPC was significantly increased in GWI subjects (brick bars) compared to GW controls (solid bars).

Analysis of the degree of unsaturation of PL classes in human subjects

It has been shown that the brain can synthesize SFA and MUFA, however most essential PUFA are acquired through the periphery due to the low capacity of the brain to synthesize these *de novo*. In order to investigate if GWI diagnosis influences unsaturation status of different PL classes, we examined SFA, MUFA and PUFA containing PL in human subjects. Figure 5-2 shows SFA, MUFA and PUFA containing species of PC, LPC, PE, LPE and PI in GWI subjects as a percentage of controls. For PI, no SFA-containing species were found.

There were no differences between veterans with GWI and controls for the degree of unsaturation for PE and PI even after adjusting for potential confounding by gender and age, respectively. However, SFA containing PC species were reduced by 22% in GWI patients compared to controls ($p=0.024$). No changes were seen in MUFA and PUFA containing PC species. In addition, the LPC, SFA, MUFA, and PUFA containing species were significantly elevated in GWI compared to controls by 16%, 15% and 23%, respectively, and remained significant after adjusting for age ($p < 0.05$). For LPE, PUFA containing species showed an increase of 50% in GWI compared to controls ($p<0.001$), whereas SFA and MUFA containing LPE species did not differ between the two groups.

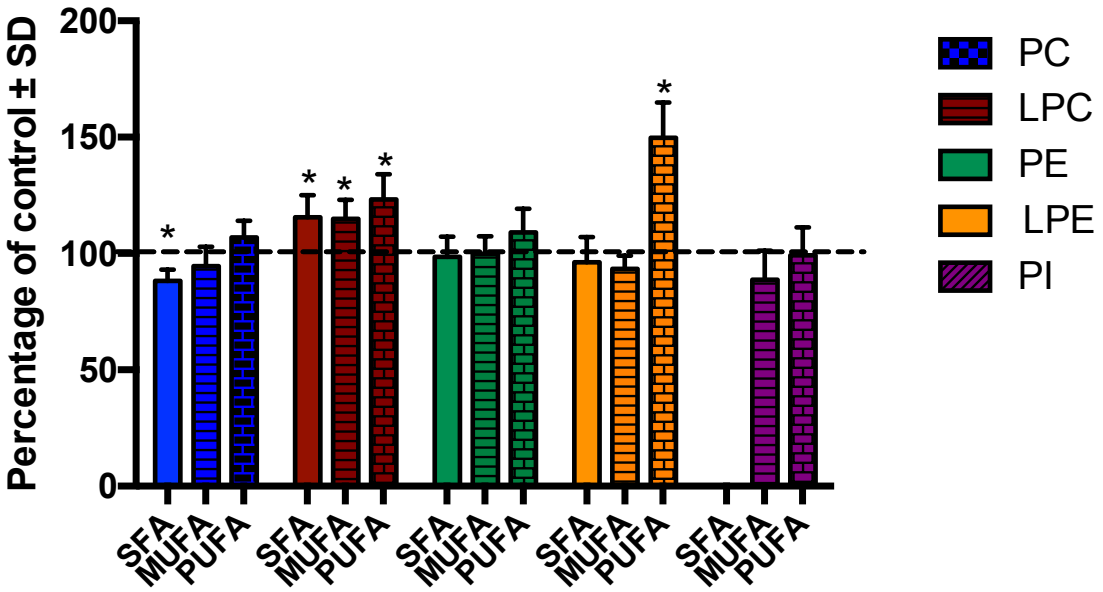


Figure 5-2 Degree of unsaturation of PL classes in plasma of GWI patients compared to controls, represented by mean ± SD as a percentage of control. For PC, SFA containing PC species were decreased in GWI patients, whereas for LPC and LPE, PUFA containing species were elevated compared to control subjects. No differences in the degree of unsaturation were observed for LPC and PE. For PI, no SFA containing species were identified (SFA: solid bars, MUFA: stripped bars, PUFA: brick bars). *$p<0.05$; MLM regression with *post hoc* analysis.

Examination of ether lipids in plasma of GWI subjects compared to controls

Ether PL are dependent upon peroxisomes for their synthesis. Therefore, we grouped ether PL within each class. Figure 5-3 shows levels of ether PC, LPC, PE and LPE in GWI subjects compared to controls. No differences were observed for ePC, eLPC and ePE between GWI and control subjects. However, eLPE levels were increased in GWI veterans by 43% relative to controls ($p<0.001$).

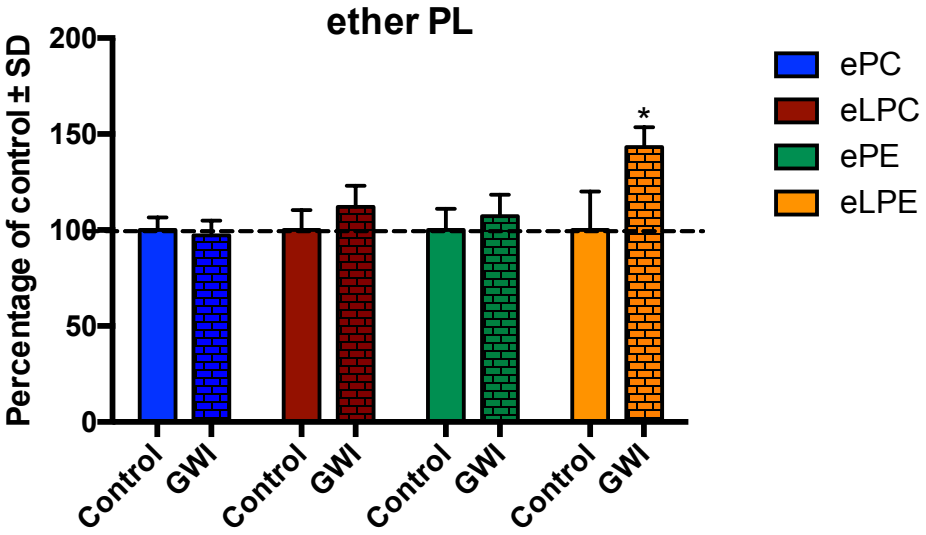


Figure 5-3 Ether lipid changes in plasma of GWI patients and compared to controls, represented by mean ± SD as a percentage of control. Levels of eLPE were significantly elevated in GWI patients (brick bars) compared to healthy GW controls (solid bars). No differences were observed for ePC, eLPC and ePE. p*<0.05; MLM regression with *post hoc* analysis.

Profiling of AA- and DHA-containing phospholipid species in GWI patients

Arachidonic acid (ω-6) and DHA (ω -3) containing fatty acids play a potential role in modulating inflammatory responses. Thus, we combined all AA and DHA containing species and AA/DHA ratios within each class for separate investigation. Figure 5-4 shows AA containing PL species of PC, LPC, PE, LPE and PI. No differences were observed for AA containing PC, PE and PI between the two groups. Compared to

controls, AA species within LPC and LPE were increased by 22% (p=0.023) and 40% (p=0.005; for LPC the p value was adjusted for potential confounding by age) in GWI compared to control subjects, respectively. For LPE, age was not detected as a cofounding factor. Figure 5-5 shows individual DHA containing PL species in the plasma of GWI subjects compared to controls. There were no differences between the two groups for DHA containing PE and PI species. However, DHA species of PC were 18% higher in GWI patients compared to controls (p=0.0013). Furthermore, within LPC and LPE, DHA containing species were increased in GWI compared to controls by 42% (p=0.001) and 91% (p<0.001), respectively (p values for PE (gender), LPC and PI (age) were adjusted as before).

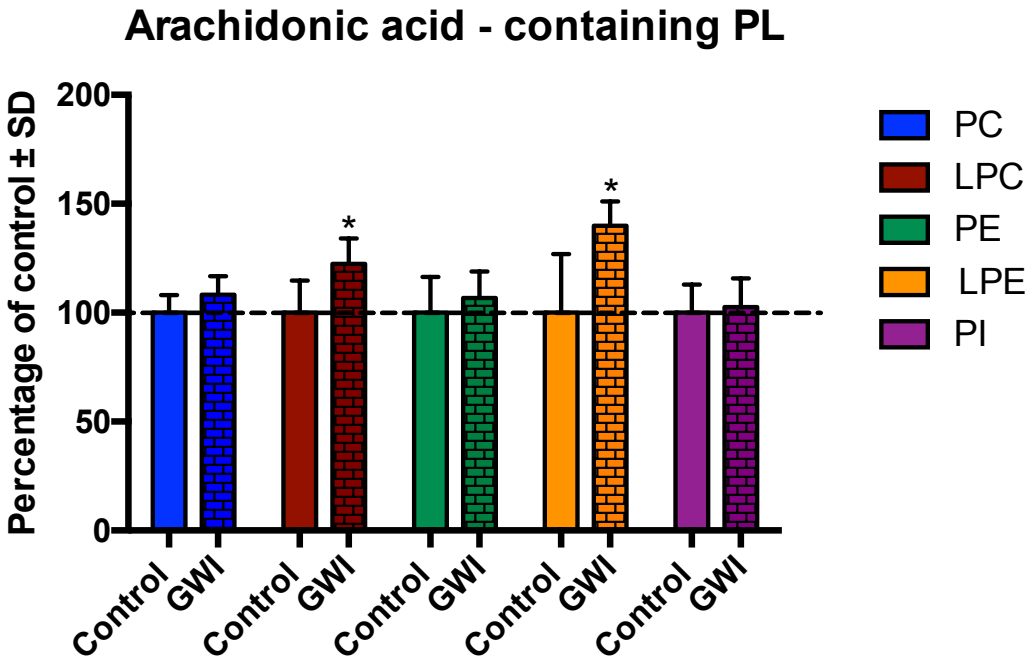


Figure 5-4 AA containing phospholipid species in plasma of GWI patients compared to controls, represented by mean ± SD as a percentage of control. Within GWI patients (brick bars), AA containing LPC and LPE species were significantly elevated compared to controls. No differences were observed within PC, PE and PI. *p<0.05; MLM regression with *post hoc* analysis.

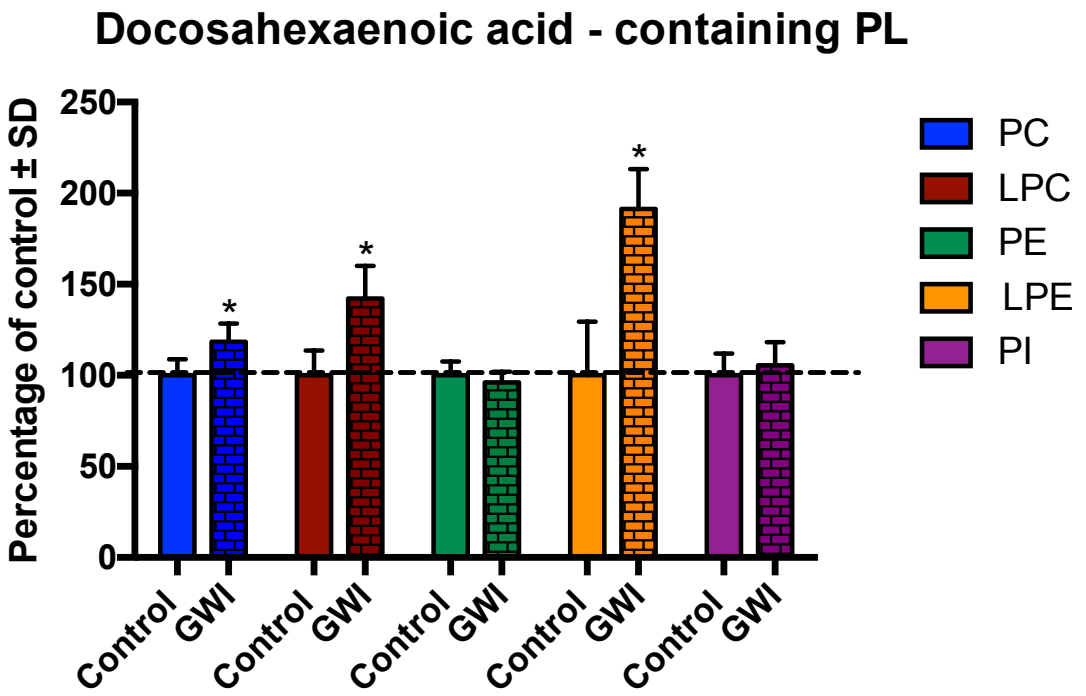


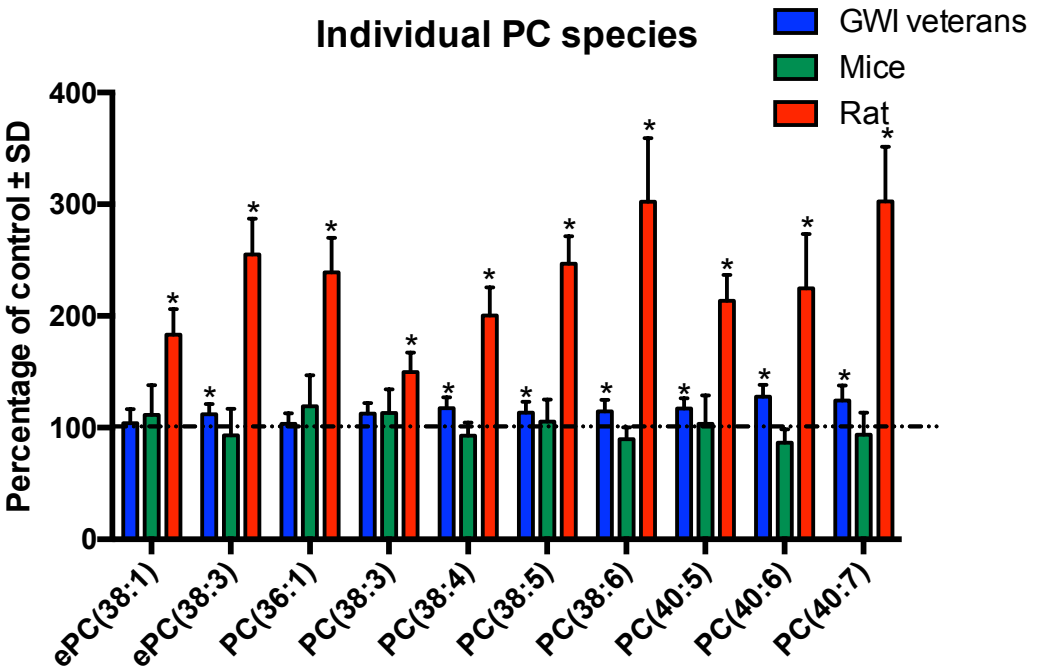
Figure 5-5 DHA containing phospholipid species in plasma of GWI patients compared to controls, represented by mean ± SD as a percentage of control. Within GWI patients (brick bars), DHA containing PC, LPC and LPE species were significantly elevated compared to controls (solid bars). No differences were observed within DHA containing PE and PI species. *p<0.05; MLM regression with *post hoc* analysis.

Comparison of individual molecular PL species across GWI rodent models and a GW veteran cohort.

In order to examine the value of the described rodent models, we investigated individual PL molecular species in the plasma of these previously published animal models of GWI (rat model: PB+PER+DEET and stress exposure, mouse model: PB+PER exposure) and compared them with the clinical findings. Total PL, degree of saturation, ether and AA and DHA content PL data for both models can be found in the Appendix, Chapter 5, Section 2 Supplement tables 1-4. For the comparison of individual molecular

species we included PL in our analysis that showed a >1.1 fold change in humans for all PL classes and examined those in the rodent GWI models.

Individual molecular species of PC that were significant for at least one model or the human subject cohort, are shown in Figure 5-6. Human GWI subjects showed significant increases in 7 out of the 10 shown individual PC species. The highest changes were observed for PC(40:6) and PC(40:7) ($p<0.05$). Increases ranged from 12% to 28%. Although the remaining 3 species showed increased levels in GWI patients, significance was not achieved. For C57Bl6/J mice PC species ePC(38:1), PC(36:1) and PC(38:3) were non significantly elevated in exposed compared to controls ($p>0.05$). As with GWI subjects, exposed rats showed significant increase of the above-mentioned PC species, as well as the remaining 3 species compared to controls ($p < 0.05$).

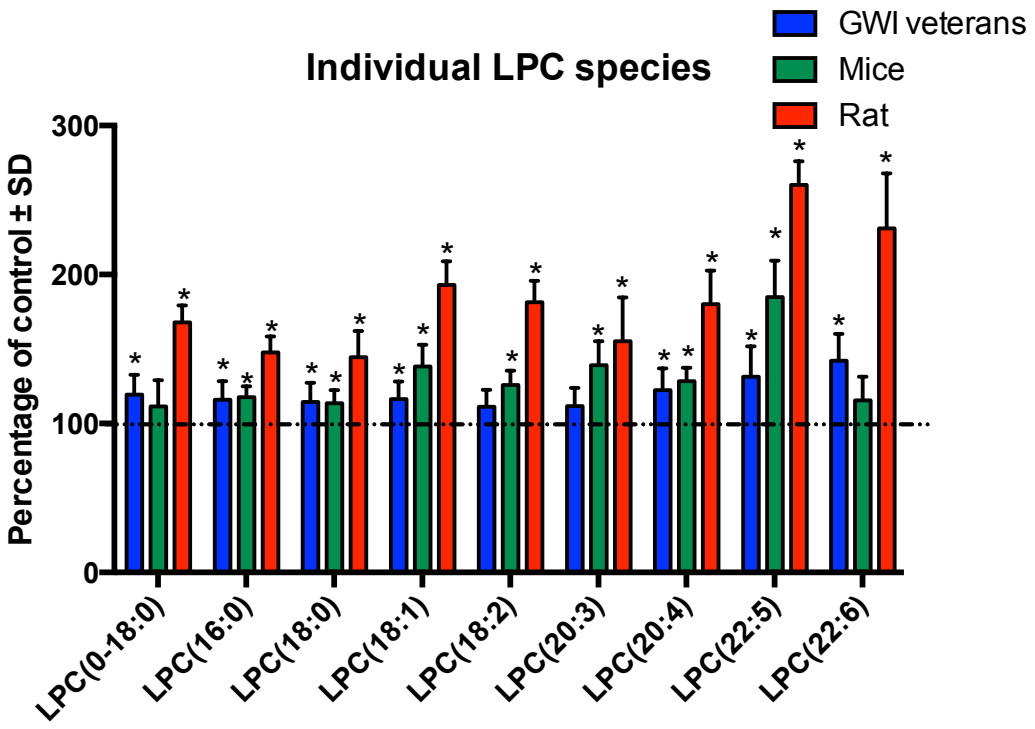


PC species	GWI veterans	Mice	Rat
ePC(38:1)			*<0.001*
ePC(38:3)	*0.04*		*<0.001*
PC(36:1)			*<0.001*
PC(38:3)			*<0.001*
PC(38:4)	*0.017*		*<0.001*
PC(38:5)	*0.043*		*<0.001*
PC(38:6)	*0.049*		*<0.001*
PC(40:5)	*0.019*		*<0.001*
PC(40:6)	*0.001*		*<0.001*
PC(40:7)	*0.011*		*<0.001*

Figure 5-6 Individual molecular species of PC are elevated in plasma from veterans with GWI, rodent model and mouse model of GWI. Blue: Human GWI plasma values expressed as a percentage of control subjects ± SD (n = 11 controls, 22 GWI) showing elevated PC species in GWI subjects compared to controls. Green: PB+PER exposed mice values expressed as a percentage of unexposed mice values ± SEM (n = 4 per group) showing that most of these species were also elevated in exposed mice but did not reach statistical significance. Red: GW agent exposed rat values expressed as a percentage of unexposed rat values ± SEM (n = 6 sham, n = 5 exposed rats) showing elevated PC species in exposed compared to control rats. *p<0.05; MLM regression with *post hoc* analysis.

Individual molecular species of LPC that were significant for at least one model or the human subject cohort, are shown in Figure 5-7. After adjusting for age, we observed

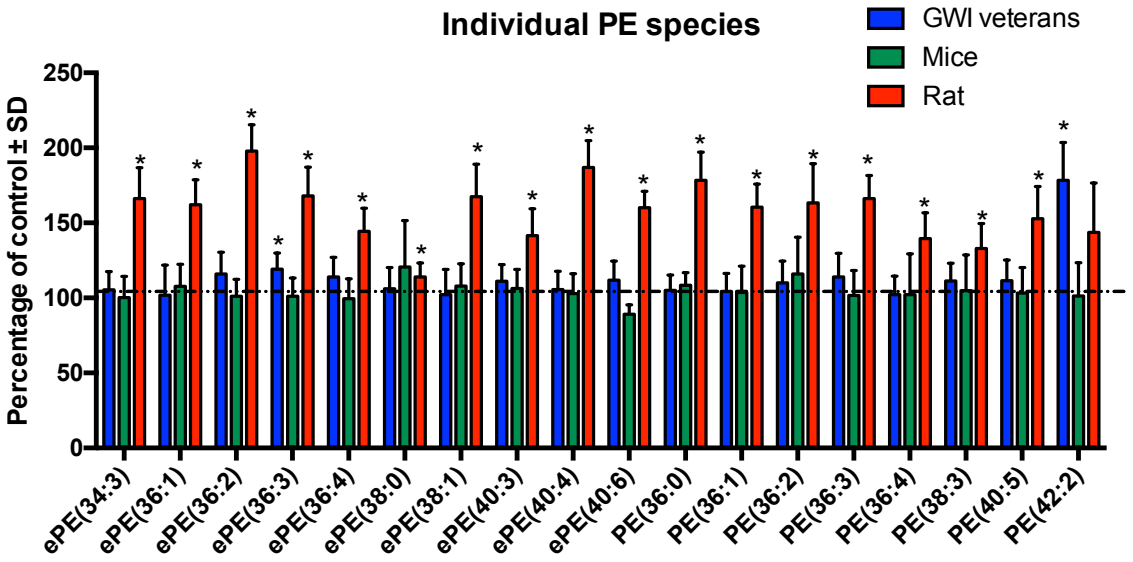
significantly increased levels for 7 out of the 9 shown molecular lipid species for the GWI patients compared to controls. Levels increased in the range of 19% (LPC(0-18:0)) to 42% (LPC(22:6n3)). Although, the remaining two LPC species (LPC(18:2n6) and LPC(20:3)) did not reach significance, a trend ($p = 0.06$-0.07) for the elevation was observed. For the GWI mouse model, statistical significance was achieved for 6 out of 9 individual molecular LPC species, including LPC(18:2n6) and LPC(20:4n6) ($p<0.05$); The remaining 3 showed a trend toward increase in the PB+PER mouse group (LPC(22:6n3), $p=0.069$, LPC(0-18:0), $p=0.071$, LPC(22:5n3), $p=0.063$). The rat GWI model showed significant increase of all shown LPC species in the exposure group ($p<0.05$), with percent change ranging from 44% (LPC(18:0)) to 160% (LPC(22:5n3)).



LPC species	GWI veterans	Mice	Rat
LPC(0-18:0)	*0.013*		*<0.001*
LPC(16:0)	*0.047*	*0.002*	*<0.001*
LPC(18:0)	*0.035*	*0.031*	*<0.001*
LPC(18:1)	*0.019*	*0.016*	*<0.001*
LPC(18:2)		*<0.001*	*<0.001*
LPC(20:3)		*0.002*	*0.041*
LPC(20:4)	*0.023*	*<0.001*	*<0.001*
LPC(22:5)	*0.028*	*0.001*	*<0.001*
LPC(22:6)	*0.001*		*<0.001*

Figure 5-7 Individual molecular species of LPC are elevated in plasma from veterans with GWI, rodent model and mouse model of GWI. Blue: Human GWI plasma values expressed as a percentage of control subjects ± SD (n = 11 controls, 22 GWI) showing elevated LPC species in GWI subjects compared to controls. Green: PB+PER exposed mice values expressed as a percentage of unexposed mice values ± SEM (n = 4 per group) showing that most of these species were also elevated in exposed mice. Red: GW agent exposed rat values expressed as a percentage of unexposed rat values ± SEM (n = 6 sham, n = 5 exposed rats) showing elevated LPC species in exposed compared to control rats. *p<0.05; MLM regression with *post hoc* analysis.

Individual molecular species of PE that were significant for at least one model or the human subject cohort, are shown in Figure 5-8. For human subjects, although we observed tendencies of elevated PE levels in the GWI patient group, only two molecular species reached significance ($p<0.05$, ePE(36:3) and PE(42:2)). Ether PE(36:3) was elevated by 19% and PE(42:2) by 78%. P values were adjusted for gender effect. Within the mouse model variance was high and no molecular species was significant ($p>0.05$). PE species ePE(38:0) and PE(36:2) were non significantly elevated. Exposed rats showed the strongest signal and significant increases compared to controls for all shown PE species except one ($p>0.05$; PE(42:2).

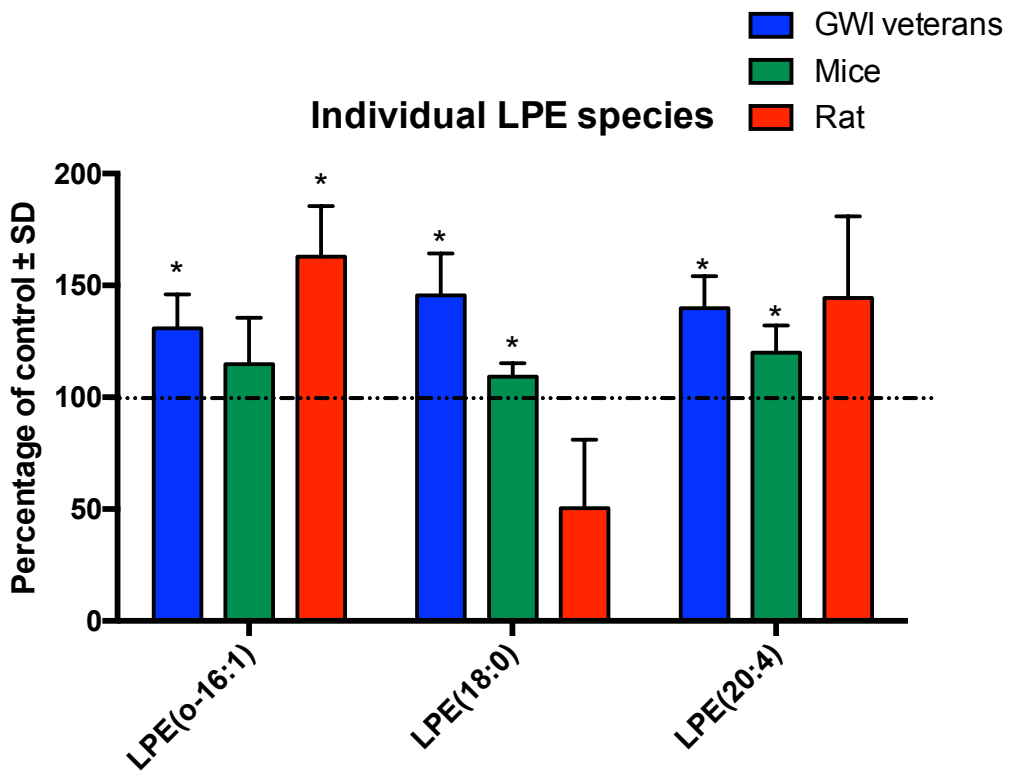


PE species	GWI veterans	Mice	Rat
ePE(34:3)			*0.002*
ePE(36:1)			*0.014*
ePE(36:2)			*<0.001*
ePE(36:3)		*0.05*	*<0.001*
ePE(36:4)			*0.002*
ePE(38:0)			*0.022*
ePE(38:1)			*0.021*
ePE(40:3)			*<0.001*
ePE(40:4)			*<0.001*
ePE(40:6)			*<0.001*
PE(36:0)			*<0.001*
PE(36:1)			*<0.001*
PE(36:2)			*<0.001*
PE(36:3)			*<0.001*
PE(36:4)			*0.004*
PE(38:3)			*0.011*
PE(40:5)			*<0.001*
PE(42:2)		*0.027*	

Figure 5-8 Individual molecular species of PE are elevated in plasma from veterans with GWI, rodent model and mouse model of GWI. Blue: Human GWI plasma values expressed as a percentage of control subjects ± SD (n = 11 controls, 22 GWI) showing two elevated PE species in GWI subjects compared to controls. Green: PB+PER exposed mice values expressed as a percentage of unexposed mice values ± SEM (n = 4 per group) showing no significant difference in PE in exposed mice. Red: GW agent exposed rat values expressed as a percentage of unexposed rat values ± SEM (n = 6 sham, n = 5 exposed rats) showing elevated PE species in exposed compared to control rats. *p<0.05; MLM regression with *post hoc* analysis.

Individual molecular species of LPE that were significant for at least one model or the human subject cohort, are shown in Figure 5-9. For LPE, all shown individual molecular

species were significantly elevated in human GWI patients compared to control ($p<0.05$, LPE(0-16:1), LPE(18:0) and LPE(20:4)), with increases between 31% for LPE(0-16:1) and 46% for LPE(18:0). The mouse model showed 2 species, which overlapped to humans that were elevated as well, namely for LPE(18:0) (9%) and LPE(20:4) (20%; $p<0.05$), whereas the rat model only showed a significant increase for LPE(0-16:1) by 63% ($p<0.05$).

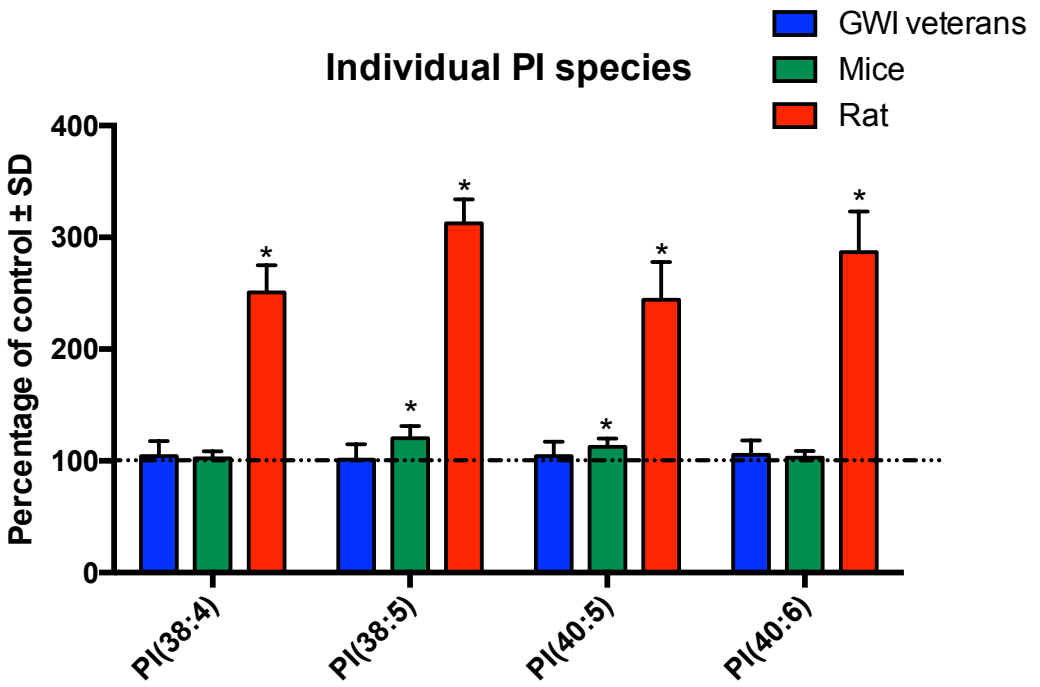


LPE species	GWI veterans	Mice	Rat
LPE(o-16:1)	*0.02*		0.007
LPE(18:0)	*0.004*	*0.012*	
LPE(20:4)	*0.005*	*0.033*	

Figure 5-9 Individual molecular species of LPE are elevated in plasma from veterans with GWI, rodent model and mouse model of GWI. Blue: Human GWI plasma values expressed as a percentage of control subjects ± SD (n = 11 controls, 22 GWI) showing elevated LPE species in GWI subjects compared to controls. Green: PB+PER exposed mice values expressed as a percentage of unexposed mice values ± SEM (n = 4 per group) showing that two species were also elevated in exposed mice. Red: GW agent exposed rat values expressed as a percentage of unexposed rat values ± SEM (n = 6 sham, n = 5 exposed

rats) showing one elevated LPE species in exposed compared to control rats. *$p<0.05$; MLM regression with *post hoc* analysis.

Individual molecular species of PI that were significant for at least one model or the human subject cohort, are shown in Figure 5-10. For PI, none of the shown individual molecular species were significantly different in human GWI patients compared to controls ($p>0.05$). The p value was adjusted for age effect. PB+PER mice showed increased levels for two out of the five PI species, PI(38:5) and PI(40:5) ($p<0.05$) by 20% and 13% respectively. Consistent with this, exposed rats showed a significant increase for all PI species ((PI38:4), PI(38:5), PI(40:5) and PI (40:6), with elevations ranging from 143% to 212%.



PI species	GWI veterans	Mice	Rat
PI(38:4)			*<0.001*
PI(38:5)		*0.025*	*<0.001*
PI(40:5)		*0.05*	*<0.001*
PI(40:6)			*<0.001*

Figure 5-10 Individual molecular species of PI are elevated in plasma from veterans, rodent model and mouse model of GWI. Blue: Human GWI plasma values expressed as a percentage of control subjects ± SD (n = 11 controls, 22 GWI) showing no differences in PI species in GWI subjects compared to controls. Green: PB+PER exposed mice values expressed as a percentage of unexposed mice values ± SEM (n = 4 per group) showing that two of these species were elevated in exposed mice. Red: GW agent exposed rat values expressed as a percentage of unexposed rat values ± SEM (n = 6 sham, n = 5 exposed rats) showing elevated PI species in exposed compared to control rats. *p<0.05; MLM regression with *post hoc* analysis.

5.4 Discussion

Shortly after returning from the 1991 conflict, a large proportion of GW veterans presented with multiple symptoms, later defined as GWI. The heterogeneous clinical presentation has made it difficult to objectively diagnose GWI, identify underlying pathology and find effective treatments for this debilitating illness. Preclinical animal models play a critical role in developing therapeutic targets. The heterogeneity of human exposure during the conflict can be addressed to some extent by looking at animal models of different GW agent exposures. These laboratory animals are more genetically homogenous than humans and environmental effects are better controlled in laboratory settings. Therefore, they allow for a more controlled assessment of the impact of GW agent exposures on brain changes that might be relevant to GWI. Identification of overlapping changes in GWI rodent models and human patients will likely facilitate development of diagnostic and therapeutic approaches that are relevant to the underlying pathological features of GWI. In this study, we performed PL profiling to identify areas of overlap between the clinical patient population and GWI mouse models.

The PB+PER mouse model and the PB+PER+DEET and stress rat model utilized in this study have been well characterized with respect to neurobehavioral features that are similar to symptoms reported by GWI patients [205,378,380,383]. In the PB+PER mouse model, this includes anxiety and cognitive deficits, which is accompanied by astroglial activation in the brains of exposed mice at 5, 16 and 22 months post exposure [378,383,384].The PB+PER+DEET+stress rat model characterized by Shetty and colleagues exhibits depressive and anxiety like behavior with spatial learning and memory deficits that are

evident at 3 months post exposure [380]. Furthermore, this model shows hippocampal pathology accompanied by decreased neurogenesis, partial loss of glutamatergic and GABA-ergic neurons, astroglial hypertrophy, microglial activation and mild inflammation [380–382]. As such, these rodent models are ideal candidates for translational studies aimed at examining long-term changes that are relevant to chronic features of GWI symptomatology. However, rodent physiology is different from human and don't fully capture the their conditions. Yet, they can be useful even if they only address certain aspects of human disease. In this case observed brain symptoms are well replicated in these rodent models, therefore we are interested in lipids in order determine if such models can provide an indication of biological changes that are relevant to the human condition.

We evaluated total PL levels for each class in plasma from GWI and control GW veterans and the two rodent models of GWI described above. We observed elevated plasma levels of total LPC in veterans with GWI compared to controls. Both mouse and rat models also showed significantly higher total LPC levels in exposed animals compared to controls. For total PC, PE and PI there were no significant differences between the groups for human and mouse studies. However, exposed rats showed a particularly strong increase in these PL classes. Because rats were exposed to stress and DEET, in addition to PB+PER, future investigation in subtypes of exposure in relation to GWI severity and symptom profiles may provide insights into possible differential response to GW agents and PL profiles.

We also examined the degree of unsaturation of different PL classes in the human cohort and in the rodent models. In general, there was a good overlap for the degree of

unsaturation of LPC, with all SFA, MUFA and PUFA being elevated in GWI patients and in the GWI animal models (see supplemental data). In particular, PUFA LPC species reached statistical significance in both rodent GWI models and in human GWI patients. There was also a significant increase in PUFA within LPE in GWI patients and although these levels were elevated in both rodent models, this difference did not reach statistical significance. In humans, we observed that SFA-containing PC species were decreased whereas MUFA and PUFA were non-significantly elevated in GWI patients and controls. In mice, although SFA and PUFA containing PC species did not differ significantly, MUFA containing species were elevated in exposed compared to control mice. In rats however, significant increases were seen for SFA, MUFA and PUFA species. Similar differences were found for PE. No significance was observed in humans, however mice showed lower SFA and PUFA containing PE species, whereas rats had increased levels of saturated and unsaturated PE. For SFA, MUFA and PUFA containing PI no significance was detected in humans and mice, however rats showed increased levels. These studies suggest possible differences in PL profiles in response to different combinations of GW exposures and chronicity of illness, which warrant further investigation. As most of these PL in plasma are bound to various lipoproteins, known differences between human and rodent lipoprotein compositions could influence these findings [385]. For example, in human plasma, initial uptake of cell cholesterol by HDL is subsequently followed by esterification through lecithin:cholesterol acyltransferase (LCAT). A portion of the HDL cholesteryl esters are then not delivered directly to the liver, but transferred by the cholesteryl ester transfer protein (CETP) to low and very low-density lipoprotein (LDL and VLDL). This is followed by a delivery of these

cholesteryl esters in the VLDL/LDL pool to the liver. Mice however, do not possess CETP. This results in the fact that while humans transport most of their plasma cholesterol in LDL, mice use HDL for this process [386]. Furthermore, HDL particles, which contain PL, differ in humans and rodents in regard to their size patterns [387]. Therefore, it may be helpful to further study the lipoprotein composition in rodents in order to understand the complexity of our findings.

It is known that SFA and MUFA can be synthesized *de novo* within the brain, yet essential PUFA are mainly supplied from the periphery [318]. For example, LPC (22:6n3) can be directly transported from plasma to the brain via the specialized mfsd2a receptor [388]. As such, increases in essential PUFA containing PL species could indicate transport deficiencies into various organs, including the brain. However, further investigation of the LPC and LPE content of the brain is necessary to fully understand the reasons behind those changes. Additionally, among PUFA, the shorter-chain ω3 and ω6 fatty acids alpha-linolenic acid (18:3n3) and linoleic acid (18:2n6) are obtained from the diet and, to some extent, can be used to synthesize their longer chain fatty acid counterparts, such as eicosatetraenoic acid (ETA 20:4n3), AA (20:4n6), eicosapentaenoic acid (EPA 20:5n3), docosapentaenoic acid (DPA 22:5n3) and DHA (22:6n3). Among these PUFA, AA and DHA containing lipid species are involved in regulating the intensity and duration of inflammatory responses [389] and possible changes in their levels could influence the immune/inflammatory imbalances observed in GWI [374]. Our findings in relation to changes in PUFA, particularly AA and DHA containing PL, suggest examination of bioactive metabolites of AA and DHA in relation to GWI pathology in the future. Owing to the potential role of AA and DHA containing species in inflammation, we examined

PL containing these fatty acids and found that AA containing PL species increased for LPC in humans, mice and rats. For LPE only humans and the mouse model reached significance, although rats showed a trend in the same direction. DHA containing species of LPC, LPE and PC were higher in GWI subjects. The same was observed for PC and LPC in rats with no DHA containing LPE species being identified in this model. However, in mice significance for these three PL classes was not achieved. For DHA containing PE and PI, no changes were observed in humans and mice, whereas levels in rats were elevated. Interestingly DHA and AA containing PC species were marginally increased in the brains of 5 month old PB+PER GWI mice [229]. However, further studies investigating lipid enzymes, transport and metabolism are necessary to understand the relationship between brain and peripheral changes. Yet, this investigation and previous studies clearly underline the importance of lipid modulation in GWI pathology [228,229,367,369].

We also observed changes in ether lipids. Within all PL classes, only eLPE showed elevation in GWI patients compared to control. Although eLPC showed an increase, significance was not achieved, owing to high variance between the samples. As ether PL are dependent upon peroxisomes for their synthesis, examination of their functionality should be investigated in the future. However, in mice no ether lipid class was significantly changed, whereas rats showed significant increases for ePC, eLPC and ePE. Again, a possibility could be that the elevated stress dose in rats leads to changes in ether lipids, which are not observed in humans or mice. It is also interesting that eLPE is only differentially regulated in humans.

We also compared individual molecular species of each PL class within rats and

mice to human plasma samples to identify species with diagnostic potential and to investigate overlap between the GWI models and veterans with this condition with the ultimate goal of facilitating future translational work aimed at developing therapies for treating GWI. Overall, we observed increases in all classes for overlapping molecular species. The most consistency was seen for several individual LPC species, such as LPC(16:0), LPC(18:0), LPC(18:1n9) and LPC(20:4n6).

Finally, the rat model showed the strongest signal with elevations reaching up to 200% for all PL classes. Compared to clinical samples, as well as the mouse model, rats received 5 minutes of restraint stress in addition to GW agents. Some epidemiological studies in GW veterans have shown that operational stress amplifies the effects of GW agents, leading to more severe symptom presentations of GWI [201,379]. These findings have been supported by exercise challenges in humans showing exercise induced hyperalgesia in a GWI subpopulation as well as alterations in immune functioning, cognition and brain structure [390]. Moreover animal studies have shown that combined GW agent and stress exposure induce long-term behavioral consequences as well as long-term learning dysfunction in a rat model of GWI [369,370]. This could explain why changes in rats show the strongest increases among all studied groups. Because of this it would be interesting to include a stress paradigm in our mouse model as well as collect plasma samples of GWI veterans before and after exercise challenge to determine if lipid metabolism is further disturbed. It is also interesting to note that we observed the most significant changes for lyso PL, LPC and LPE, within the GWI patients compared to controls. Since LPE and LPC can be generated by phospholipase action on PC and fatty acyl CoA can reacylate lyso-PC to form PC future studies should examine enzyme activities involved in

PL metabolism and remodeling. Furthermore, peripheral changes could reflect brain lipid changes, which would implement disturbances in lipid transport and therefore should be examined. Alternatively, if plasma lipid disturbances do not reflect brain changes, these abnormalities could reflect immune dysfunction in GWI (i.e. autoimmune responses, inflammation) and would be another area of interest to investigate. Finally, the study of lipoprotein content (HDL, VLDL etc.) could shed further light on the involvement of phospholipids in GWI.

Although no differences in PC and PE concentration were observed in humans, increases in LPC and LPE could indicate increased phospholipase A2 (PLA2) activity. Furthermore, the acyltransferase activities (LPCAT3 and LPCAT4), which convert LPC and LPE to PC and PE respectively, could be affected. More investigation is needed to explain the observed changes. Finally, major PI species showed no effect in response to GW agent exposure in humans.

As these were pilot studies, there are number of limitations present which do not fully allow us to assess potential use of lipid changes in diagnosing GWI. Our human sample size was small and therefore we were not able to fully assess the impact of gender and age on PL levels in this case control study, but instead we adjusted our statistical analyses to account for these confounding factors. Additionally, in order to assess diagnostic strength validation in another cohort is necessary. We are not yet able to generalize our findings to GWI in the veteran population with validation being required in a larger cohort to accomplish this. Validation studies using a larger cohort of GWI and control GW veterans will also allow us the opportunity to assess differential response to various combinations of exposure to GW agents and to investigate differences in lipid profiles

based on the severity of symptoms and different symptom clusters in order to identify subgroups of veterans with GWI. This could ultimately improve our ability to provide an objective diagnosis and management of relevant symptoms in subgroups of ill veterans.

5.5 Conclusion

In this study, we identified significantly different overlapping phospholipid changes between two different rodent models of GWI and a clinical sample of veterans with GWI. These differences were particularly evident within the lysophospholipids. This first attempt at a pilot translational study using two different rodent models and a clinical cohort of veterans with GWI proved successful in suggesting strong potential avenues for further larger studies of phospholipid biomarkers of GWI. These results also provide evidence for further study of these models for therapeutic target discovery if validated in larger preclinical and clinical samples.

Chapter 6 Discussion

6.1 Summary of thesis research

Traumatic brain injury is a debilitating illness, affecting over 1.7 million people in the US per year, with 5.3 million experiencing long-term consequences. As most TBIs are categorized as mild (~80%), diagnosis, monitoring and treatment have been difficult. [18,391]. Furthermore, its high prelevance in the military together with its comorbidity with PTSD has made it clear that an objective measure is needed that can address exposure and symptom heterogeneity. In the case of GWI it has been almost 25 years since the Persian Gulf War and the veterans' exposure to Gulf War agents. Although symptom categories and clusters are used to identify veterans with GWI, there are no available biomarkers to assist with diagnosis. The self-report system of GWI comes with a large reporting bias, given the many years, which have elapsed since the exposure, and the lack of objective data on the use of PB, PER and DEET, which has made it difficult to pin point the origin of this debilitating disease. Therefore, many GW veterans exhibit GWI symptoms but lack a formal diagnosis due to the absence of an objective measure. Therefore in order to objectively diagnose veterans with *any* of these conditions, biomarkers (or panels of biomarkers) are needed.

Protein biomarkers have been widely studied, especially in TBI, yet mostly at acute and sub-acute phases post injury. These biomarkers have shown great value at immediate hospital admissions. For soldiers that are deployed acute care is available, however acute diagnostics and the ability to evaluate biomarkers might not be accessible, especially in cases of mTBI. Furthermore, for illnesses such as PTSD it is

crucial to develop a measurement that can diagnose the illness without self-reporting bias. Thus, in this thesis I first and foremost investigated plasma samples from 120 human active-duty soldiers with a history of TBI (mTBI), PTSD and/or both compared to control individuals in order to identify chronic peripheral changes of these conditions (Chapter 2 & Chapter 3). Assessments were made via patient reported outcomes (PRO). Whilst clinician-reported outcomes are important and are often used in in clinical trials, in which a physician or clinician records observer outcomes-related data, based on their interpretation of the treatment's efficacy. This can include for examples results on brain imaging, drug measurements in blood or tumor size in cancer patients. However this approach fails to take into account patient reported outcomes. The U.S. Food & Drug Administration defines PROs as "Any report coming directly from patients… about a health condition and its treatment.[392]" Relying on clinician reported outcomes alone, results are only seen as in trial participants as a groups. Yet, individual PRO can help to develop personalized treatment, which might differ among subjects due to similar but varying underlying biology of their condition.

6.1.1 Proteomic studies

In chapter 2 we focused our investigation on global protein profiling via liquid chromatography mass spectrometry to detect changes in protein content. Although none of the 3 identified proteins, for which there was evidence of differential expression, passed multiple test correction threshold, the validation experiment demonstrated a significant increase in the TBI+PTSD population compared to controls. Although, mostly

known to be differently expressed in certain cancer types, LRG1 expression was found to be induced in a mice and model [283].

This is of interest as comorbidity of TBI and PTSD has been under recent focus. For example a study by Haarbauer-Krupa et. al. investigated the occurrence of PTSD 6 months post mTBI in a civilian population. They observed that the incidence of PTSD after 6 months was 27% for mTBI subjects who had screened negative for PTSD at the time of injury assessment [393]. In military personnel that have a history of TBI the PTSD incidence numbers are even higher. It has been estimated that PTSD develops following mTBI in 32-66% of military cases [28]. Therefore we believe that LRG1 could be of value in clinical assessment and could be used as a potential diagnostic tool for the identification of TBI and PTSD comorbidity at chronic stages.

Furthermore, we used a CHI single mTBI mouse model to see if similar changes occurred in the plasma of these animals. Although we did not observe significant differences in LRG1 concentrations at the acute 24hrs timepoint post injury, we were able to see a non-significant trend ($p=0.06$) for increased LRG1 concentrations in mTBI mice compared to controls at 3 months post injury. At 6 months post injury LRG1 levels were significantly elevated in mTBI animals. At the later timepoints of 12 and 24 months post injury levels of injured mice stayed elevated, however LRG1 was also increased in control animals. Therefore the effect of age on LRG1 levels meant significance was lost.

How LRG1 contributes to TBI and PTSD pathology is unclear. However, it has been shown that increased LRG1 expression promotes angiogenesis via TGFβ1 endothelial cell signaling [359]. Multiple studies have observed that angiogenesis plays an important role in mediating functional recovery after TBI (reviewed by [360]). Furthermore,

if LRG1 influences TGFβ1 signaling, the protein could have influence on a variety of TGFβ1 effector responses, such as inflammation [394]. Although major regulators of angiogenesis such as $CD34^+$ EPCs and VGFR have been shown to return to baseline days after TBI, LRG1 responses could be continuing as a secondary mechanism. In fact, our clinical cohort was comprised of individuals who had sustained a mild TBI and PTSD, but showed good recovery, as their participation in our study was during their preparation for their upcoming deployment. Thus, it is possible that LRG1 is a positive indicator for outcome in these conditions. Therefore further studies should include subjects with poor outcome after injury to investigate if their LRG1 levels are unchanged. If that is the case then future studies could investigate LRG1 as a potential drug target to induce angiogenesis and possible other responses. Furthermore, inhibition of LRG1 or LRG1 knockout mice could be used in a model of TBI and/or PTSD to investigate outcome compared to control or wild type animals respectively.

In conclusion, although more investigation is needed on how LRG1 is connected to TBI, we believe that LRG1 holds an interesting potential for the detection of TBI and PTSD at chronic timepoints after injury.

After performing global proteomics, and targeted protein analysis of LRG1, our next interest was to see if the investigated conditions would have long-term effects on peripheral lipid levels.

6.1.2 Lipidomic studies

In the past decade lipids have become a focal point for more attention in conjunction with TBI, as well as PTSD and GWI, due to their high abundance in the

brain and the fact that they can cross the blood-brain barrier more readily than proteins [322].

Altered lipid metabolism has been shown to contribute to CNS injury [298] and abnormal phospholipid (PL) profiles have been reported in the CSF of patients with TBI [299]. Studies on PTSD patients showed increases in cholesterol, low-density lipoproteins, triglycerides, and a reduction of high-density lipoproteins in blood [300–303]. Thus, in chapter 3 we continued our investigation on the same military cohort by examining plasma lipid abnormalities. Additionally, soldiers were genotyped for apolipoprotein E (APOE) due to the connection between the ApoE protein, lipid transport and TBI. We observed changes in concentration of 6 major PL classes. Those classes (PC, LPC, PE, LPE, PI and SM) were significantly decreased in TBI, PTSD and TBI+PTSD compared to controls, with signal strength (least to strongest decrease) in the following order PTSD<TBI<TBI+PTSD compared to controls. We examined degree of saturation and showed that MUFA containing PC and PI species were lower in the TBI and TBI+PTSD groups. However these PLs were unaltered among PTSD subjects compared to controls. Similarly, ether PC levels were lower in PTSD and TBI+PTSD subjects relative to controls. Ratios of AA to DHA containing species were significantly decreased within PC and PE classes. APOE ε4 (+) subjects exhibited higher PL levels than their APOE ε4 (-) counterparts within the same diagnostic groups. Lastly, PTSD severity analysis revealed that significant PL decreases were primarily restricted to the moderate-to-severe PTSD group.

We created a ROC curve for each diagnostic stemming from multiple individual lipid species, determining their sensitivity and specificity. It is important to mention that

the changes observed in peripheral lipids were relatively small, although significant. For total lipids changes ranged in the order PTSD (10~20%) <TBI (20~30%) <TBI+PTSD (25~40%). These rather small changes are not unusual for lipids in disease, as shown by other groups in the field of lipidomics [395,396]. We recommend that findings should be replicated in a larger cohort.

Although findings of our lipidomic findings need to be validated, we have shown that PL alterations are evident in individuals with chronic mild TBI and PTSD.

After the discovery of lipid involvement at chronic stages post-TBI in our population, we investigated plasma lipid content of those mentioned phospholipid classes in one of our well characterized mouse models of mTBI.(Mouzon et al. 2012; Mouzon et al. 2014; Mouzon *pers. comm.*) (Chapter 4) to determine if our clinical cohort findings could be replicated preclinically. As animal samples were collected at an acute time point (24 hrs post-injury) and at several chronic stages (3, 6, 12 and 24 months post-injury) from injured mice and sham controls we were able to perform plasma phospholipid profiling over this 2-year period post-injury. Overall, statistical analysis extending over multiple timepoints revealed that lipid levels were not different at 24hrs post injury but changed over the next 3 months as indicated by the decrease in mTBI versus control at the 3 months timepoint. We then observed a possible recovery phase around the 6 months timepoint, where PL levels returned to those of control animals. Finally, lipid levels dropped again at the chronic 12- and 24 months timepoints post injury. The reason behind these changes could be increased oxidation of phospholipids. At the moment we are in the process of developing an MS method to measure these oxidative lipids, but were not able to implementing this approach for this work. Therefore, we performed a

standard lipid peroxidation assay that showed significantly increased oxidation in TBI animals at 3months compared to 24hrs, where no difference between control and TBI mice was observed. Once our oxidative lipid MS assay is ready we hope to perform measurements not only on the animal cohort but on human subjects as well.

In correlation to the military cohort, we were able to reproduce our findings in the animal model showing that at chronic time points lower levels of several major PL classes in mTBI mice compared to controls were observed. We further showed that certain lipid species are affected by age more than others. For example, SFA, MUFA and PUFA fatty acids were differentially regulated over time. Ether containing PE species were elevated at 24 hrs post-injury and decreased relative to controls at chronic stages. Arachidonic acid (AA) and docosahexaenoic acid (DHA) containing species were significantly decreased within all PL classes at the chronic stages. We are aware that the military cohort is a cross-sectional analysis, whereas lipid levels were measured at several timepoints post injury in mice. Therefore, we hope to use a similar approach in human cohorts in the near future as we did in animals to see how lipid levels behave over a time course of acute to subacute and chronic injury phases.

As we were able to show that a lipidomic platform has great potential for the investigation of biomarker profiles we used this approach for another debilitating disease, namely Gulf War Illness. Furthermore, previous global proteomics by our group in a mouse model of GWI had revealed alteration in lipid metabolism [367].

In chapter 5 we included healthy Gulf War Veterans and veterans with GWI and compared their lipid profiles to mouse and rat models of GWI. We observed increases of

multiple phospholipid (PL) species across all studied cohorts. Furthermore, our data suggested dysfunction within ether -and DHA and AA-containing PL species, supporting the proposed role for immune and inflammatory balance with veterans with GWI. Although the animal models were able to reproduce lipid observation in humans it was interesting to observe the different levels of changes in lipid signals between the models. This emphasizes the need for more comparative studies among models. Whereas the mouse model and human samples showed similar levels of increases (1.2~1.5 fold) in lipid levels for GWI, the rat model exceeded these elevated values in some cases by 2-3 fold. It is of note that the rat samples provided by Dr. Shetty also included, besides PB+PER exposure (also used in mice) a stress paradigm and use of DEET in the model. Furthermore, our sample size for this human cohort was small (N=33). Therefore our findings need to be replicated in other cohorts.

6.2 Limitations and future directions

Human samples are influenced by various factors that are not typically found in corresponding animal models. In humans the severity of injury or trauma varies due to different exposures. In the case of TBI, injury can vary in impact strength, type of impact (blast/penetrating) and brain area of impact between individuals. Additionally, gender, age and ethnic background all have been shown to influence biological signals as well as disease progression and outcome. Although age and gender matched groups are routinely compared, ethnicity is often overlooked and ethnic groups are frequently not equally represented. Furthermore, others and we have shown that genetic variants, such as those encoded by the APOE gene, can influence experimental data and therefore need to be

accounted for. In this study we stratified our analysis by APOE ε4carrier and non-carrier, due to the involvement of the protein in lipid transport and its known influence on TBI outcome, but other genes could also influence our outcome measures, such as genes innvolved in the HPA axis stress response. It would be therefore beneficial for future analysis to include relevant genetic polymorphisms as cofactors. Because of the heterogeneity of the TBI/PTSD cohort a higher number of individuals should be sampled. Furthermore lifestyle habits, including diet, smoking, alcohol consumption and exercise regime can all influence biological outcome measurements and many of these factors rely on self-report. Although it is almost impossible to control perfectly for all these factors, active duty military personnel stationed at Fort Riley and Camp Shelby received similar diets (plus samples were collected during fasting) and have comparable fitness levels. Moreover, use of medication in patients at the time of sample collection can interfere with scientific findings, especially in cases where severe symptoms are present and need to be treated. In our study use of medication was reduced to a minimum, as active duty soldiers were getting ready for a one-year deployment, therefore passing medical examination. Furthermore, for the majority of subjects the upcoming deployment was their first military assignment, therefore individuals with TBI and/or PTSD received these exposures in a non combat environment, mostly during training. Together all described factors add to the heterogeneity of samples, making validation across clinical cohorts difficult and we suggest validation of our findings in multiple larger and diverse cohorts.

Currently, our scientists at the Roskamp Institute are working with the Tampa VA and collecting blood samples from subjects with mild TBI. Additionally, at the moment we are working on collaborations with the Boston and Bronx VA, where we expect to

receive a high number of samples from veterans with GWI and relevant controls. We hope to use those samples once collection is complete to target the same protein/lipids observed in this study.

Furthermore, whilst the assessment of levels of molecules is a good way to gain information about the association of such molecules in a disease state, other approaches should be used in the future to understand the underlying mechanism that connects the molecular changes with the condition. One concept could investigate enzymatic activity in relations to the investigated molecule. Additionally, the location of the molecule could contribute to its role in the disease; therefore studying certain regions (e.g. brain regions) and specific cell types can share light on the function of this molecule. Moreover, solubility (membrane bound or not) of a molecule can be important. Finally, posttranslational modification (PTM), reversible or irreversible chemical alterations of a protein after its translation, play a great role in protein function. Examples of protein modification include chemical groups (e.g. phosphoryl), lipids (e.g. palmitic acid), carbohydrates (e.g. glucose) or even entire proteins (e.g. ubiquitin) to amino acid side chains, as well as the enzymatic cleavage of peptide bonds [397]. Most PTM occur at side chains that can act as either strong (C, M, S, T, Y, K, H, R, D, E) or weak (N, Q) nucleophiles, leading to changes in protein dynamics, binding affinity and structure which can affect protein function. Therefore, future studies should investigate changes in regards to these mechanisms to understand why certain biomarkers are found in the mentioned conditions.

Some of the mentioned confounding factors can be avoided in animal models. Controlled standard diet, age and gender matched cloned lines of mice together with identical housing conditions allow researchers to minimize variables. Moreover, genetically manipulated mice or rats afford the opportunity to explore the influence of genetic risk factors. Animal studies also allow easier implementation longitudinal study paradigms (as shown in Chapter 4). The animal model we used for comparison with our TBI military cohort is a single mTBI model. Our Institute is also working with a repetitive mTBI (r-mTBI) model. At the moment a r-mTBI + PTSD (and PTSD alone) model is being developed by Ph.D. student Moustafa Algamal, who will compare our existing r-mTBI model with the r-mTBI +PTSD and PTSD mice in regards to behavior, pathology, proteomics and lipidomics analyses.

The measured plasma lipid concentrations observed in the aforementioned conditions, as well as LRG1, serve as the basis for a panel of objective biomarkers; therefore they do not necessarily reflect the pathological underlying mechanism. However, animal models could be used in the future to link the observed changes to alterations of specific biological mechanisms.

Given the complexity and diversity of lipids, which has started recently to receive much attention, different approaches are needed to fully investigate variations in the biological responses within different lipid classes. Approaches for the measurement of lipids includes two major categories: LC- based or shotgun MS. In our studies, we used LC-based MS due to the complexity of lipids and their highly dynamic range in plasma. Our LC-based lipidomic approach reduces the complexity of PL, by allowing each class of lipid to elute based on the polarity of the head group and chain length of fatty acids

within each class. This achieves enrichment of low abundance species and the identification of many individual species. Whilst shotgun lipidomics is a high-throughput approach it comes at the expense preserving the complexity of a lipid extract due to the absence of pre-chromatographic separation [398].

Our lipidomics studies indicate peripheral chronic lipid disturbances for the investigated conditions (overall decreases in TBI/PTSD and increases in GWI). However, it should be noted that PL only represent a subclass class of lipids. Therefore future investigations need to extend these analyses and also examine other classes such as sterols, sphingolipids including cardiolipin and cholesterol. Furthermore, we need to understand the pathological role of lipids, therefore in is crucial to examine their relevance in relation to their metabolites, such as the PUFA's eicosanoids, including prostaglandins, thromboxanes and leukotrienes. Furthermore, as mentioned previously, PUFAs are prone to oxidations; therefore measurement of oxidative lipids is necessary and will be done in the near future.

Another approach should investigate the state of PLs in plasma. It is known that PE, PI, and SM are only present bound to lipoproteins, whereas PC and LPC can be found on both lipoproteins and plasma lipoprotein free fraction (PLFF)[399]. Furthermore, apolipoproteins are associated with very low-density lipoproteins (VLDL), intermediate density lipoproteins, chylomicron remnants, and certain subclasses of high-density lipoproteins (HDL). It has been shown that in terms of the distribution of apoE among the various plasma lipoproteins, apoE4 has a preference for large, triglyceride-rich VLDL particles, whereas apoE3 and apoE2 associate with the small, phospholipid- rich HDL.

As a result lipoprotein fraction should be investigated to gain deeper understanding about PL metabolism in our cohorts. Similar, plasma exosomes, which are bioactive vesicles originating from multivesicular bodies, have shown to contain high levels of PC (59%), SM (21%) and PE (9%)[400]. Playing an important role in communication between cells, exosomes transport lipids and lipolytic enzymes. They are of specific interest as therapeutic drugs could be conveyed by encapsulation into these liposomes. It has been suggested that exosomes and the knowledge of their lipid composition/organization could be used as the first "therapeutic vehicles" in order to treat various diseases[401]. Therefore, future lipidomics studies should investigate exosomes in the plasma of our patient groups, as well as their phospholipid composition to possibly find a link between the physiology of PL and lipoprotein/vesicle bodies presentation.

In order to establish our observed lipid changes as a biomarker more work is needed. The biomarker needs to be shown to be valid and reliable under clinical trial conditions and has to show a strong relationship to clinical outcome. Therefore, our findings need to be repeated in other cohorts. Finally, once a biomarker is established targeted data analysis or exploration of other approaches is necessary to improve throughput, suitable for routine clinical work.

DHA is known to promote hippocampal neurogenesis in the adult brain [402,403], modulate signal transduction molecules and receptor affinity [404] and plays a role in the support of mitochondrial functioning [405]. Furthermore, DHA is known to reduce inflammatory cytokines (interleukin IL-1, tumor necrosis factor, TNF) [406] and reactive oxygen species [407], as well as exhibit anti-inflammatory properties (see discussion

Chapter 3-5) [319].Evidence for the beneficial effects of DHA supplementation in TBI comes from various preclinical studies, which have been extensively reviewed [408]. It has been shown that long chain PUFA supplementation before or after TBI in mice protect the brain by restoring the expression of specific protective mediators, limiting structural damage to the axon and events such as neuronal apoptosis, and diminishing TBI induced cognitive dysfunction [408]. As both animal models and human studies investigating lipid involvement in TBI suggest that n-3 supplementation would be beneficial two new clinical trials are currently recruiting (https://clinicaltrials.gov/). Miller et al. at the University of Texas Southwestern Medical Center aim to use 2g of DHA treatment daily for 3 month in patients with concussion due to sports-related injury (mTBI) (https://clinicaltrials.gov/ct2/show/NCT01903525). Another new study by Bica et al. at East Carolina University aims to investigate concussion outcome after treatment with high dose of n-3 fatty acid containing 2200mg of DHA for 30 days after onset of concussion symptoms (https://clinicaltrials.gov/ct2/show/NCT01814527).

Due to the fact that DHA has been linked with hippocampal neurogenesis, it has been suggested that n-3 supplementation during the early post-trauma period could facilitate the clearance of fear memory from the hippocampus and consequently minimize PTSD symptoms [409]. Clinical studies have supported this hypothesis, as PTSD symptoms were shown to be lower in DHA treated PTSD injured patients compared to placebos [410]. Furthermore, a clinical trial investigating PTSD symptoms in female rescue workers who were deployed during the acute disaster phase of the Great East Japan Earthquake showed attenuated PTSD symptoms [411]. Although not all trials were able to confirm these positive findings, higher n-3 intake has been linked to other mood disorders, such as

major depressive disorder and bipolar disorder [412–414] and thus the importance of lipid homeostasis in these conditions cannot be ignored.

As more evidence arises for the involvement of lipid dysfunction in Gulf War Illness it is only a matter of time until investigation of long-chain PUFA supplementation in Gulf War veterans move into clinical trials. Furthermore, GWI has been linked with mitochondrial dysfunction [230]. The connection of mitochondria and lipid homeostasis has been extensively reviewed [415] and thus, looking forward, investigation of isolated mitochondria and changes in their lipid content could add to the understanding of the underlying pathology of GWI.

Statistics

Data generated by Mass Spectrometry leads to the problem of multicollinearity during analysis, meaning in a multiple regression model two or more predictor variables are highly correlated and they can be linearly predicted from the others with a great degree of accuracy. Multicollinearity in big data analytics can lead to biased estimation and variance inflation. In order to address this problem, different statistical approaches can be used, including avoiding multiple comparisons via univariant analysis with relaxed p values followed by confirmation set (used for our proteomic data set), or multiple comparisons via bootstrapping methods, PCA (used for our lipidomic data set) and machine learning methods such as random forest/trees tests.

One of the most common ways to deal with multicollinearity is *Principal Component Analysis*. In PCA, orthogonal transformations are used to remove correlations

variables into an output set of values of linearly uncorrelated variables (named principal components). Although the technique is sensitive to hypothesis testing, missing data, outliners, transformations, inaccuracy if out outcome variables were greater than our sample size as well as its assumption of normal distribution of data, PCA is robust when used for description, meaning high in accuracy as long as data is continuous, does not have to many outliners and is not strongly skewed[416].

The bootstrapping method relies on resampling sample data. In general, random samples are drawn from a full sample set and estimates of the standard errors are computed for each. After that an average of all the bootstrap samples is computed. The jackknifing approach is similar to the bootstrapping method, but making use of recomputing the standard error, each time eliminating one case. Then, an average of the standard errors is used. Although this method is relatively simply using standard errors and confidence intervals, checks for stability of data it does not well deal with the issue of multicollinearity nor allows the reduction of the dimensions of our dataset, as assumptions are being made in this statistical methods (such as independence of samples)[416].

Machine learning is a concept of computer science and artificial intelligence. It aims to build systems (algorithms), which learn from data, in contrast to programming instructions. Random forest/trees tests are an example of machine learning approaches, where random subsets of variables for each tree are chosen and the most frequent tree output is used as the overall classification. Random forest itself does not remove multicollineraity, but works well if the prediction with a validation and predicting set has the same of collinearity. Therefore, this approach is often used discovery and validation

sets. Since we are in the discovery phase of this project, this statistical method will be of value in the future, when we validate our findings[416].

Multicollinearity can be avoided by only observing one variable at a time (univariant analysis), therefore not making multiple comparisons. Since all our data set contained multiple variables we chose to use mixed model ANOVA for our independent proteomic data.

Overall different approaches can be used for statistical evaluation of our generated data sets. The used tests in this thesis have proven strong analytical strength by other groups in the field, as well as our groups at Roskamp Institute.

The work described in this thesis represents a new approach in the field of biomarker discovery. It extends the existing literature with a focus on biomarkers in TBI to chronic phases of injury, indicating that even in mild TBI secondary injury mechanisms persist over a long period of time. Our findings that show proteomic and lipid abnormalities at these late timepoints could facilitate development of diagnostics especially for the military, where immediate admission to a well equipped hospital might not always be possible. Furthermore, the chronic and heterogenic illnesses PTSD and GWI, where diagnosis relies heavily on self-report are in dire need for an objective panel of biomarkers. Additionally, a biomarker panel could be used for personalized care and help monitor illness development and treatment response. This thesis is the first step in such a direction. Findings in our animal models show that they are well fitted for further characterization and expansion of TBI, PTSD and GWI research areas. Our studies provide information on biochemical abnormalities that persist long after the initial

injury/trauma event and exposure to chemical reagents; these abnormalities may provide useful insight into the continuing pathogenesis and serve as preliminary data for the development of diagnostic markers or drug targets for these diseases.

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APPENDIX

Chapter 2

Section 1

Chapter 2 Table 1: Mass Spectrometry approaches

Approach	Description	Benefits	Limitations
1.Stable Isotope-Labeling	***Peptide sequence is labeled with chemically equivalent mass tag; Label keeps biochemical/ analytical properties of the peptide but changes its mass. Thus, can be used as a relative reference, as different samples can be combined but is still distinguishable in MS analysis.***		
1.1 Metabolic labeling: Stable isotope labeling with amino acids in cell culture (SILAC) (Ong et al., 2002)	Heavy isotopes (2H, 13C or 15N) are introduced to a cell culture; cell turnover incorporates them only into newly synthesized proteins	Use of cell or organism; no limits of amount of sample to be labeled	Expensive, no analysis of human samples possible, the labeled arginine can become a proline
1.2 Chemical labeling	***Isotopes are introduced chemically at protein/peptide level***		
Dimethyl labeling (Boearseman 2009)	Tryptic digests are labeled with sodium cyanoborohydride (NaBH3CN) to generate "light" label (+28 Da per primary amine) and sodium cyanoborodeuteride ($NaBD_3CN$) to methylate amines to generate "heavy" label (+36 Da per primary amine).	Cost-effective, fast reaction, can be used for a varieties of biological samples; can work with small amounts of sample (μg - mg range), reliable;	Small isotope effect during LC separation due to deuterated labels.
^{18}O labeling (1951 by Sprinson and Rittenberg)	Protease (e.g. trypsin) catalyzes exchange of two ^{16}O atoms for two ^{18}O atoms at the C-terminal carboxyl group of proteolytic peptides; results in mass shift of 4 Da between differently labeled peptides	Simple, low cost	Occurrence of incomplete incorporation of two ^{18}O atoms into peptide leads to difficulties in data analysis
Isotope-coded affinity tags (ICAT) (Gygi et al., 1999)	Stable isotope-bearing chemical reagents are introduced to reactive sites (cysteine) on protein/peptide. Reagent consists of a reactive group that is cysteine-directed, a polyether linker region with 8 deuteriums and a biotin group that allows purification of labeled peptides. Denaturation and reduction of	Limited peptide mixture complexity (only cysteine-containing peptides are isolated), which can enable identification of lower abundant proteins	Proteins could contain no cysteines or only in single peptide; large biotin tag increases fragmentation complexity of spectra

	two pools of proteins results in derivatization of cysteine residues with either the 'heavy' or 'light' ICAT reagent.		
Isobaric tags for relative and absolute quantitation (iTRAQ) (Ross et al., 2004)	Labeling of N-termini and lysine side chains with different isobaric mass reagent; Different mass tags only distinguishable after peptide fragmentation; Each tag adds the same total mass to a given peptide results in each peptide species producing a single peak during LC	Analysis of any sample possible; Multiplexing of up to 8 samples without increased complexity at MS analysis	Expensive, unstable chemical reagents; Introduction of isotope label at peptide level; MS with low m/z measuring capabilities necessary; peptide quantification depends on a single tandem mass spectrum
Isobaric labeling (tandem mass tags (TMT) (Thompson et al., 2003)		Analysis of any sample possible; Multiplexing of up to 10 samples without increased complexity at MS analysis	
2. Label-free quantification	**Direct analysis of protiens in samples, no mixing of samples, comparison after analysis**	**High coverage of proteome; no limit to number of experiments**	**Lack of a formal internal standard, leads to error in individual datasets**

Section 2 - Neurocognitive Measures

PTSD Checklist-Military Version (PCL-M)

Instruction to patient: Below is a list of problems and complaints that veterans sometimes have in response to stressful life experiences. Please read each one carefully, put an "X" in the box to indicate how much you have been bothered by that problem *in the last month*.

No.	Response	Not at all (1)	A little bit (2)	Moderatly (3)	Quite a bit (4)	Extremely (5)
1.	Repeated, disturbing *memories, thoughts, or images* of a stressful military experience from the past?					
2.	Repeated, disturbing *dreams* of a stressful military experience from the past?					
3.	Suddenly *acting* or *feeling* as if a stressful military experience *were happening* again (as if you were reliving it)?					
4.	Feeling *very upset* when *something reminded* you of a stressful military experience from the past?					
5.	Having *physical reactions* (e.g., heart pounding, trouble breathing, or sweating) when *something reminded* you of a stressful military experience from the past?					
6.	Avoid *thinking about* or *talking about* a stressful military experience from the past or avoid *having feelings* related to it?					
7.	Avoid *activities* or *situations* because they *remind you* of a stressful military experience from the past?					
8.	Trouble *remembering important parts* of a stressful military experience from the past?					
9.	Loss of *interest in things that you used to enjoy?*					
10.	Feeling *distant* or *cut* off from other people?					
11.	Feeling *emotionally numb* or being unable to have loving feelings for those close to you?					
12.	Feeling as if your *future* will somehow be *cut short*?					
13.	Trouble *falling* or *staying asleep*?					
14.	Feeling *irritable* or having *angry outbursts*?					
15.	Having *difficulty concentrating*?					
16.	Being *"super alert"* or watchful on guard?					
17.	Feeling *jumpy* or easily startled?					

PCL-M for DSM-IV (11/1/94) Weathers, Litz, Huska, & Keane National Center for PTSD - Behavioral Science Division

Life Events Checklist

	Event	Happened to me	Witnessed it	Learned about it	Not Sure	Doesn't apply
1.	Natural disaster (for example, flood, hurricane, tornado, earthquake)					
2.	Fire or explosion					
3.	Transportation accident (for example, car accident, boat accident, train wreck, plane crash)					
4.	Serious accident at work, home, or during recreational activity					
5.	Exposure to toxic substance (for example, dangerous chemicals, radiation)					
6.	Physical assault (for example, being attacked, hit, slapped, kicked, beaten up)					
7.	Assault with a weapon (for example, being shot, stabbed, threatened with a knife, gun, bomb)					
8.	Sexual assault (rape, attempted rape, made to perform any type of sexual act through force or threat of harm)					
9.	Other unwanted or uncomfortable sexual experience					
10.	Combat or exposure to a war-zone (in the military or as a civilian)					
11.	Captivity (for example, being kidnapped, abducted, held hostage, prisoner of war)					
12.	Life-threatening illness or injury					
13.	Severe human suffering					
14.	Sudden, violent death (for example, homicide, suicide)					
15.	Sudden, unexpected death of someone close to you					
16.	Serious injury, harm, or death you caused to someone else					
17.	Any other very stressful event or experience					

3 Question DVBIC TBI Screening Tool

1. Did you have any injury(ies) during your deployment from any of the following? (check all that apply):

A. ☐ Fragment
B. ☐ Bullet
C. ☐ Vehicular (any type of vehicle, including airplane)
D. ☐ Fall
E. ☐ Blast (Improvised Explosive Device, RPG, Land mine, Grenade, etc.)
F. ☐ Other specify: _____

2. Did any injury received while you were deployed result in any of the following? (check all that apply):

A. ☐ Being dazed, confused or "seeing stars"
B. ☐ Not remembering the injury
C. ☐ Losing consciousness (knocked out) for less than a minute
D. ☐ Losing consciousness for 1-20 minutes
E. ☐ Losing consciousness for longer than 20 minutes

NOTE: Endorsement of A-E meets criteria for positive TBI Screen

F. ☐ Having any symptoms of concussion afterward (such as headache, dizziness, irritability, etc.)
G. ☐ Head Injury

NOTE: Confirm F and G through clinical interview

H. ☐ None of the above

3. Are you currently experiencing any of the following problems that you think might be related to a possible head injury or concussion? (check all that apply):

A. ☐ Headaches
B. ☐ Dizziness
C. ☐ Memory problems
D. ☐ Balance problems
E. ☐ Ringing in the ears
F. ☐ Irritability
G. ☐ Sleep problems
H. ☐ Other specify:_____

Schwab, K. A., Baker, G., Ivins, B., Sluss-Tiller, M., Lux, W., & Warden, D. (2006). The Brief Traumatic Brain Injury Screen (BTBIS): Investigating the validity of a self-report instrument for detecting traumatic brain injury (TBI) in troops returning from deployment in Afghanistan and Iraq. *Neurology, 66*(5)(Supp. 2), A235.

For more information contact:
Telephone: 1-800-870-9244 Email: info@DVBIC.org Web: www.DVBIC.org

Zung Depression Scale (ZDS)

Please read each statement and decide how much of the time the statement describes how you've been feeling during the past **2 weeks**. Respond to all statements.

Make check mark (✓) in appropriate column.	**A little of the time**	**Some of the time**	**Good part of the time**	**Most of the time**
1. I feel down-hearted and blue				
2. Morning is when I feel the best				
3. I have crying spells or feel like it				
4. I have trouble sleeping at night				
5. I eat as much as I used to				
6. I still enjoy sex				
7. I notice that I am losing weight				
8. I have trouble with constipation				
9. My heart beats faster than usual				
10. I get tired for no reason				
11. My mind is as clear as it used to be				
12. I find it easy to do the things I used to				
13. I am restless and can't keep still				
14. I feel hopeful about the future				
15. I am more irritable than usual				
16. I find it easy to make decisions				
17. I feel that I am useful and needed				
18. My life is pretty full				
19. I feel that others would be better off if I were dead				
20. I still enjoy the things I used to do				

Adapted from: Zung, W.W. (1965). A Self-Rating Depression Scale. *Archives of General Psychiatry, 12*: 63-70.

Zung Anxiety Scale (ZAS)

For each item below, please place a check mark (✓) in the column which best describes how often you felt or behaved this way during the past several days. Bring the completed form with you to the office for scoring and assessment during your office visit.

Place check mark (✓) in correct column.	A little of the time	Some of the time	Good part of the time	Most of the time
1 I feel more nervous and anxious than usual.				
2 I feel afraid for no reason at all.				
3 I get upset easily or feel panicky.				
4 I feel like I'm falling apart and going to pieces.				
5 I feel that everything is all right and nothing bad will happen.				
6 My arms and legs shake and tremble.				
7 I am bothered by headaches neck and back pain.				
8 I feel weak and get tired easily.				
9 I feel calm and can sit still easily.				
10 I can feel my heart beating fast.				
11 I am bothered by dizzy spells.				
12 I have fainting spells or feel like it.				
13 I can breathe in and out easily.				
14 I get feelings of numbness and tingling in my fingers & toes.				
15 I am bothered by stomach aches or indigestion.				
16 I have to empty my bladder often.				
17 My hands are usually dry and warm.				
18 My face gets hot and blushes.				
19 I fall asleep easily and get a good night's rest.				
20 I have nightmares.				

Source: William W.K. Zung. A rating instrument for anxiety disorders. Psychosomatics. 1971

Alcohol Use Identification Test (Audit)

Date: _____

ALCOHOL USE DISORDERS IDENTIFICATION TEST (AUDIT)

Please circle the answer that is correct for you.

1. How often do you have a drink containing alcohol?

Never	Monthly or less	Two to four times a month	Two to three times a week	Four or more times a week

2. How many drinks containing alcohol do you have on a typical day when you are drinking?

1 or 2	3 or 4	5 or 6	7 to 9	10 or more

3. How often do you have six or more drinks on one occasion?

Never	Less than monthly	Monthly	Weekly	Daily or almost daily

4. How often during the last year have you found that you were not able to stop drinking once you had started?

Never	Less than monthly	Monthly	Weekly	Daily or almost daily

5. How often during the last year have you failed to do what was normally expected from you because of drinking?

Never	Less than monthly	Monthly	Weekly	Daily or almost daily

6. How often during the last year have you needed a first drink in the morning to get yourself going after a heavy drinking session

Never	Less than monthly	Monthly	Weekly	Daily or almost daily

7. How often during the last year have you had a feeling of guilt or remorse after drinking?

Never	Less than monthly	Monthly	Weekly	Daily or almost daily

8. How often during the last year have you been unable to remember what happened the night before because you had been drinking?

Never	Less than monthly	Monthly	Weekly	Daily or almost daily

9. Have you or someone else been injured as a result of your drinking?

No	Yes, but not in the last year	Yes, during the last year

10. Has a relative or friend, or a doctor or other health worker been concerned about your drinking or suggested you cut down?

No	Yes, but not in the last year	Yes, during the last year

REFERENCE

Saunders, J. B., Aasland, O. G., Babor, F., et al. (1993). Development of the alcohol use disorders screening test (AUDIT). WHO collaborative project on early detection of persons with harmful alcohol consumption, II. Addiction, 88, 791-804.

Epworth Sleepiness Scale

Name: _____ Today's date: _____

Your age (Yrs): _____ Your sex (Male = M, Female = F): _____

How likely are you to doze off or fall asleep in the following situations, in contrast to feeling just tired?

This refers to your usual way of life in recent times.

Even if you haven't done some of these things recently try to work out how they would have affected you.

Use the following scale to choose the **most appropriate number** for each situation:

0 = would **never** doze
1 = **slight chance** of dozing
2 = **moderate chance** of dozing
3 = **high chance** of dozing

It is important that you answer each question as best you can.

Situation	**Chance of Dozing (0-3)**
Sitting and reading _____	___
Watching TV _____	___
Sitting, inactive in a public place (e.g. a theatre or a meeting) _____	___
As a passenger in a car for an hour without a break _____	___
Lying down to rest in the afternoon when circumstances permit _____	___
Sitting and talking to someone _____	___
Sitting quietly after a lunch without alcohol _____	___
In a car, while stopped for a few minutes in the traffic _____	

THANK YOU FOR YOUR COOPERATION

PITTSBURGH SLEEP QUALITY INDEX (PSQI)

INSTRUCTIONS: The following questions relate to your usual sleep habits during the past month only. Your answers should indicate the most accurate reply for the majority of days and nights in the past month. Please answer all questions.

1. During the past month, when have you usually gone to bed at night?

   USUAL BED TIME_____

2. During the past month, how long (in minutes) has it usually take you to fall asleep each night?

   NUMBER OF MINUTES_____

3. During the past month, when have you usually gotten up in the morning?

   USUAL GETTING UP TIME_____

4. During the past month, how many hours of actual sleep did you get at night? (This may be different than the number of hours you spend in bed.)

   HOURS OF SLEEP PER NIGHT_____

INSTRUCTIONS: For each of the remaining questions, check the one best response. Please answer all questions.

5. During the past month, how often have you had trouble sleeping because you...

	Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
(a) ...cannot get to sleep within 30 minutes	☐	☐	☐	☐
(b) ...wake up in the middle of the night or early morning	☐	☐	☐	☐
(c) ...have to get up to use the bathroom	☐	☐	☐	☐
(d ...cannot breathe comfortably	☐	☐	☐	☐
(e) ...cough or snore loudly	☐	☐	☐	☐
(f) ...feel too cold	☐	☐	☐	☐
(g) ...feel too hot	☐	☐	☐	☐
(h) ...had bad dreams	☐	☐	☐	☐
(i) ...have pain	☐	☐	☐	☐
(j) Other reason(s), please describe				
How often during the past month have you had trouble sleeping because of this?	☐	☐	☐	☐

PSQI Page 1

	Very good	Fairly good	Fairly bad	very bad
6. During the past month, how would you rate your sleep quality overall?	☐	☐	☐	☐

	Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
7. During the past month, how often have you taken medicine (prescribed or "over the counter") to help you sleep?	☐	☐	☐	☐
8. During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?	☐	☐	☐	☐

	No problem at all	Only a very slight problem	Somewhat of a problem	A very big problem
9. During the past month, how much of a problem has it been for you to keep up enough enthusiasm to get things done?	☐	☐	☐	☐

	No bed partner or roommate	Partner/ roommate in other room	Partner in same room, but not same bed	Partner in same bed
10. During the past month, how much of a problem has it been for you to keep up enough enthusiasm to get things done?	☐	☐	☐	☐

If you have a roommate or bed partner, ask him/her how often in the past month you have had...

	Not during the past month	Less than once a week	Once or twice a week	Three or more times a week
(a) ...loud snoring	☐	☐	☐	☐
(b) ...long pauses between breaths while asleep	☐	☐	☐	☐
(c) ...legs twitching or jerking while you sleep	☐	☐	☐	☐
(d) ...episodes of disorientation or confusion during sleep	☐	☐	☐	☐
(e) Other restlessness while you sleep; please describe	☐	☐	☐	☐

_____

_____

SCORING INSTRUCTIONS FOR THE PITTSBURGH SLEEP QUALITY INDEX:

The Pittsburgh Sleep Quality Index (PSQI) contains 19 self-rated questions and 5 questions rated by the bed partner or roommate (if one is available). Only self-rated questions are included in the scoring. The 19 self-rated items are combined to form seven "component" scores, each of which has a range of 0-3 points. In all cases, a score of "0" indicates no difficulty, while a score of "3" indicates severe difficulty. The seven component scores are then added to yield one "global" score, with a range of 0-21 points, "0" indicating no difficulty and "21 " indicating severe difficulties in all areas.

Scoring proceeds as follows:

Component 1: Subjective sleep quality

Examine question #6, and assign scores as follows:

Response	Component 1 score
"Very good"	0
"Fairly good"	1
"Fairly bad"	2
"Very bad"	3

Component 1 score: _____

Component 2: Sleep latency

1. Examine question #2, and assign scores as follows:

Respo\nse	Score
≤15 minutes	0
16-30 minutes	1
31-60 minutes	2
> 60 minutes	3

Question #2 score: _____

2. Examine question #5a, and assign scores as follows:

Response	Score
Not during the past month	0
Less than once a week	1
Once or twice a week	2
Three or more times a week	3

Question #5a score: _____

3. Add #2 score and #5a score

Sum of #2 and #5a: _____

4. Assign component 2 score as follows:

Sum of #2 and #5a	Component 2 score
0	0
1-2	1
3-4	2
5-6	3

Component 2 score: _____

Component 3: Sleep duration

Examine question #4, and assign scores as follows:

Response	Component 3 score
> 7 hours	0
6-7 hours	1
5-6 hours	2
< 5 hours	3

Component 3 score: _____

Component 4: Habitual sleep efficiency

1. Write the number of hours slept (question #4) here: _____
2. Calculate the number of hours spent in bed:

   Getting up time (question #3): _____

   Bedtime (question #1): _____

   _____

   Number of hours spent in bed: _____

3. Calculate habitual sleep efficiency as follows:

   (Number of hours slept/Number of hours spent in bed) X 100 = Habitual sleep efficiency (%)

   (_____/_____) X 100 = %

4. Assign component 4 score as follows:

Habitual sleep efficiency %	Component 4 score
> 85%	0
75-84%	1
65-74%	2
< 65%	3

Component 4 score: _____

Component 5: Step disturbances

1. Examine questions #5b-5j, and assign scores for each question as follows:

Response	Score
Not during the past month	0
Less than once a week	1
Once or twice a week	2
Three or more times a week	3

5b score: _____

5c score: _____

5d score: _____

5e score: _____

5f score: _____

5g score: _____

5h score: _____

5i score: _____

5j score: _____

2. Add the scores for questions #5b-5j:

Sum of #5b-5j: _____

3. Assign component 5 score as follows:

Sum of #5b-5j	Component 5 score
0	0
1-9	1
10-18-4	2
19-27	3

Component 5 score: _____

Component 6: Use of sleeping medication

Examine question #7 and assign scores as follows:

Response	Component 6 score
Not during the past month	0
Less than once a week	1
Once or twice a week	2
Three or more times a week	3

Component 6 score: _____

Component 7: Daytime dysfunction

1. Examine question #8, and assign scores as follows:

Response	Score
Never	0
Once or twice	1
Once or twice each week	2
Three or more times each week	3

Question#8 score: _____

2. Examine question #9, and assign scores as follows:

Response	Score
No problem at all	0
Only a very slight problem	1
Somewhat of a problem	2
A very big problem	3

Question #9 score: _____

3. Add the scores for question #8 and #9:

Sum of #8 and #9: _____

4. Assign component 7 score as follows:

Sum of #8 and #9	Component 7 score
0	0
1-2	1
3-4	2
5-6	3

Component 7 score: _____

Global PSQI Score

Add the seven component scores together:

Global PSQI Score: _____

Central Nervous System Vital Signs® (CNS VS)

CNS Vital Signs is comprised of eight common neuropsychological measures. The battery generates 15 primary scores, which are used to calculate 5 domain (index) scores (Memory, Psychomotor Speed, Reaction Time, Cognitive Flexibility, and Complex Attention).

Descriptions of the CNS Vital Signs measures.

Measure	Description
Verbal Memory	**Measure:** How well subject can recognize, remember, and retrieve words e.g. exploit or attend literal representations or attribute. **Relevance:** Remembering, turning off the stove, recalling an appointment or rehabilitation information, taking medications, and attending class.
Visual Memory	**Measure:** How well subject can recognize, remember and retrieve geometric figures e.g. exploit or attend symbolic or spatial representations. **Relevance:** Remembering graphic instructions, navigating, operating machines, recalling images, remember a calendar of events, and using a graphical user interface.
Composite Memory	**Measure:** How well subject is able to recognize, remember and retrieve words and geometric figures. Identify problems with the storage, manipulation, and retrieval of information. **Relevance:** Remembering and learning new information, turning off the stove, recalling an appointment or rehabilitation information, taking medications, attending class, inability to navigate in familiar places, recalling images, etc.
Processing Speed	**Measure:** How well a subject can automatically and fluently perform relatively easy or over-learned cognitive tasks, especially when high mental efficiency is required i.e. attention and focused concentration. **Relevance:** Medication effect, ability to respond/react to threats, take evasive action or see possible danger/risk signs, or issues with accuracy and detail.
Executive Function	**Measure:** How well a subject recognizes set shifting (mental flexibility) and abstraction (rules, categories) and manages multiple tasks simultaneously. **Relevance:** Ability to sequence tasks and manage multiple tasks simultaneously, flexibility required for self-correction, tracking and responding to a set of simple instructions.
Psychomotor Speed	**Measure:** How well a subject recognizes and processes information i.e., perceiving, attending/responding to incoming information, motor speed, fine motor coordination, and visual-perceptual ability.

Measure	Description
	Relevance: Distractibility, fitness-to-drive, occupation issues, obsessive concern with accuracy and detail. May be a result of: medication effects, anxiety, learning disabilities, visual perceptual problems, working under time pressure, typing and machine operation.
Reaction Time	**Measure:** How fast the subject can react, in milliseconds, to a simple and increasingly complex direction set. **Relevance:** Driving a car, attending to conversation, tracking and responding to a set of simple instructions, taking longer to decide what response to make.
Complex Attention	**Measure:** How well the subject can maintain focus and perform quickly and accurately e.g. problem attending to multiple stimuli at the same time. Ability to track and respond to information over brief or lengthy periods of time and/or performs mental tasks quickly and accurately requiring vigilance. **Relevance:** Self-regulation, learning, productivity, and behavioral control.
Cognitive Flexibility	**Measure:** How well subject is able to adapt to rapidly changing and increasingly complex set of directions and/or to manipulate the information. **Relevance:** Reasoning, switching tasks, decision-making, impulse control, strategy formation, attending to conversation.

Section 3

Chapter 2: Tables 2 - 5: ROC tables, containing all values for sensitivity and 1-specificity

TBI								
X	*Prob*	*1-Specificity*	*Sensitivity*	*Sens-(1-Spec)*	*True Pos*	*True Neg*	*False Pos*	*False Neg*
		0	0	0	0	52	0	21
77.336	0.9541	0	0.0476	0.0476	1	52	0	20
75.60559	0.9441	0	0.0952	0.0952	2	52	0	19
75.60278	0.9441	0	0.1429	0.1429	3	52	0	18
71.2181	0.9088	0	0.1905	0.1905	4	52	0	17
71.008	0.9067	0	0.2381	0.2381	5	52	0	16
70.3535	0.8998	0	0.2857	0.2857	6	52	0	15
69.84545	0.8941	0	0.3333	0.3333	7	52	0	14
64.8725	0.8227	0	0.381	0.381	8	52	0	13
62.79685	0.7833	0	0.4286	0.4286	9	52	0	12
61.30013	0.7511	0	0.4762	0.4762	10	52	0	11
61.0575	0.7456	0	0.5238	0.5238	11	52	0	10
57.62667	0.6598	0	0.5714	0.5714	12	52	0	9
57.5785	0.6585	0	0.619	0.619*	13	52	0	8
56.6405	0.6327	0.0385	0.619	0.5806	13	50	2	8
56.158	0.6191	0.0577	0.619	0.5614	13	49	3	8
53.83586	0.5513	0.0769	0.619	0.5421	13	48	4	8
52.94684	0.5247	0.0769	0.6667	0.5897	14	48	4	7
52.579	0.5137	0.0962	0.6667	0.5705	14	47	5	7
52.08271	0.4987	0.1154	0.6667	0.5513	14	46	6	7
50.8274	0.461	0.1346	0.6667	0.5321	14	45	7	7
47.79394	0.3725	0.1346	0.7143	0.5797	15	45	7	6
47.70158	0.3699	0.1538	0.7143	0.5604	15	44	8	6
46.21511	0.3293	0.1538	0.7619	0.6081	16	44	8	5
43.64446	0.2648	0.1731	0.7619	0.5888	16	43	9	5
42.6081	0.2413	0.2115	0.7619	0.5504	16	41	11	5
42.224	0.2329	0.2115	0.8095	0.598	17	41	11	4
41.643	0.2207	0.2308	0.8095	0.5788	17	40	12	4
40.9645	0.2069	0.25	0.8095	0.5595	17	39	13	4
40.64873	0.2008	0.2692	0.8095	0.5403	17	38	14	4
40.33297	0.1947	0.2885	0.8095	0.5211	17	37	15	4
39.438	0.1784	0.3077	0.8095	0.5018	17	36	16	4
39.11849	0.1728	0.3269	0.8095	0.4826	17	35	17	4
38.402	0.1608	0.3462	0.8095	0.4634	17	34	18	4
38.15	0.1568	0.3654	0.8095	0.4441	17	33	19	4

36.218	0.1284	0.3846	0.8095	0.4249	17	32	20	4
35.78863	0.1227	0.3846	0.8571	0.4725	18	32	20	3
35.3115	0.1167	0.3846	0.9048	0.5201	19	32	20	2
34.65	0.1087	0.4038	0.9048	0.5009	19	31	21	2
34.4365	0.1063	0.4231	0.9048	0.4817	19	30	22	2
33.803	0.0992	0.4423	0.9048	0.4625	19	29	23	2
33.56425	0.0967	0.4615	0.9048	0.4432	19	28	24	2
33.0987	0.0919	0.4808	0.9048	0.424	19	27	25	2
32.41859	0.0853	0.5	0.9048	0.4048	19	26	26	2
32.1965	0.0832	0.5192	0.9048	0.3855	19	25	27	2
31.68133	0.0786	0.5192	0.9524	0.4332	20	25	27	1
31.54416	0.0774	0.5192	1	0.4808	21	25	27	0
31.29016	0.0753	0.5385	1	0.4615	21	24	28	0
31.2515	0.075	0.5577	1	0.4423	21	23	29	0
29.90461	0.0645	0.5769	1	0.4231	21	22	30	0
29.316	0.0603	0.5962	1	0.4038	21	21	31	0
28.20838	0.0532	0.6154	1	0.3846	21	20	32	0
27.9332	0.0515	0.6346	1	0.3654	21	19	33	0
27.61315	0.0497	0.6538	1	0.3462	21	18	34	0
27.30021	0.0479	0.6731	1	0.3269	21	17	35	0
26.48787	0.0437	0.7115	1	0.2885	21	15	37	0
25.84419	0.0405	0.7308	1	0.2692	21	14	38	0
24.241	0.0337	0.75	1	0.25	21	13	39	0
24.04282	0.0329	0.7692	1	0.2308	21	12	40	0
22.96182	0.029	0.7885	1	0.2115	21	11	41	0
22.82823	0.0286	0.8077	1	0.1923	21	10	42	0
22.1886	0.0265	0.8269	1	0.1731	21	9	43	0
22.00162	0.0259	0.8462	1	0.1538	21	8	44	0
21.89138	0.0256	0.8654	1	0.1346	21	7	45	0
21.82021	0.0254	0.8846	1	0.1154	21	6	46	0
21.8155	0.0254	0.9038	1	0.0962	21	5	47	0
21.707	0.025	0.9423	1	0.0577	21	3	49	0
21.22106	0.0236	0.9615	1	0.0385	21	2	50	0
16.674	0.0138	0.9808	1	0.0192	21	1	51	0
12.51322	0.0084	1	1	0	21	0	52	0
12.51322	0.0084	1	1	0	21	0	52	0

Chapter 2 Table 2.

PTSD								
X	*Prob*	*1-Specificity*	*Sensitivity*	*Sens-(1-Spec)*	*True Pos*	*True Neg*	*False Pos*	*False Neg*
		0	0	0	0	52	0	34
67.2245	0.822	0	0.0294	0.0294	1	52	0	33
66.58806	0.8156	0	0.0588	0.0588	2	52	0	32
61.58237	0.7591	0	0.0882	0.0882	3	52	0	31
60.40432	0.7443	0	0.1176	0.1176	4	52	0	30
59.031	0.7262	0	0.1471	0.1471	5	52	0	29
56.6405	0.6929	0.0385	0.1471	0.1086	5	50	2	29
56.158	0.6859	0.0577	0.1765	0.1188	6	49	3	28
54.397	0.6597	0.0577	0.2059	0.1482	7	49	3	27
53.963	0.6531	0.0577	0.2353	0.1776	8	49	3	26
53.83586	0.6511	0.0769	0.2353	0.1584	8	48	4	26
52.94684	0.6373	0.0769	0.2647	0.1878	9	48	4	25
52.6995	0.6334	0.0769	0.2941	0.2172	10	48	4	24
52.579	0.6315	0.0962	0.2941	0.198	10	47	5	24
52.51299	0.6305	0.0962	0.3235	0.2274	11	47	5	23
52.08271	0.6237	0.1154	0.3235	0.2081	11	46	6	23
50.8274	0.6036	0.1346	0.3235	0.1889	11	45	7	23
48.61169	0.5672	0.1346	0.3529	0.2183	12	45	7	22
47.70158	0.552	0.1538	0.3529	0.1991	12	44	8	22
45.99	0.5233	0.1538	0.3824	0.2285	13	44	8	21
45.89864	0.5217	0.1538	0.4118	0.2579	14	44	8	20
45.3285	0.5121	0.1538	0.4706	0.3167	16	44	8	18
45.003	0.5066	0.1538	0.5	0.3462*	17	44	8	17
43.64446	0.4836	0.1731	0.5	0.3269	17	43	9	17
42.6081	0.4661	0.2115	0.5	0.2885	17	41	11	17
41.94013	0.4549	0.2115	0.5294	0.3179	18	41	11	16
41.643	0.4499	0.2308	0.5294	0.2986	18	40	12	16
40.9645	0.4386	0.25	0.5294	0.2794	18	39	13	16
40.796	0.4358	0.25	0.5588	0.3088	19	39	13	15
40.64873	0.4333	0.2692	0.5588	0.2896	19	38	14	15
40.33297	0.4281	0.2885	0.5588	0.2704	19	37	15	15
39.438	0.4133	0.3077	0.5588	0.2511	19	36	16	15
39.1755	0.409	0.3077	0.5882	0.2805	20	36	16	14
39.11849	0.4081	0.3269	0.5882	0.2613	20	35	17	14
38.82555	0.4033	0.3269	0.6176	0.2907	21	35	17	13
38.402	0.3965	0.3462	0.6176	0.2715	21	34	18	13
38.15	0.3924	0.3654	0.6176	0.2523	21	33	19	13
37.95663	0.3893	0.3654	0.6471	0.2817	22	33	19	12
36.218	0.3617	0.3846	0.6471	0.2624	22	32	20	12

35.78676	0.355	0.3846	0.6765	0.2919	23	32	20	11
34.65	0.3376	0.4038	0.7353	0.3314	25	31	21	9
34.4365	0.3344	0.4231	0.7353	0.3122	25	30	22	9
33.803	0.3249	0.4423	0.7353	0.293	25	29	23	9
33.56425	0.3214	0.4615	0.7353	0.2738	25	28	24	9
33.0987	0.3145	0.4808	0.7353	0.2545	25	27	25	9
32.7845	0.31	0.4808	0.7647	0.2839	26	27	25	8
32.41859	0.3047	0.5	0.7647	0.2647	26	26	26	8
32.1965	0.3015	0.5192	0.7647	0.2455	26	25	27	8
31.29016	0.2888	0.5385	0.7941	0.2557	27	24	28	7
31.2515	0.2882	0.5577	0.8529	0.2952	29	23	29	5
31.066	0.2857	0.5577	0.8824	0.3247	30	23	29	4
29.90461	0.2699	0.5769	0.8824	0.3054	30	22	30	4
29.316	0.2621	0.5962	0.8824	0.2862	30	21	31	4
28.20838	0.2479	0.6154	0.8824	0.267	30	20	32	4
27.9332	0.2444	0.6346	0.8824	0.2477	30	19	33	4
27.61315	0.2405	0.6538	0.8824	0.2285	30	18	34	4
27.30021	0.2366	0.6731	0.8824	0.2093	30	17	35	4
26.68501	0.2292	0.6731	0.9118	0.2387	31	17	35	3
26.48787	0.2268	0.7115	0.9118	0.2002	31	15	37	3
26.164	0.223	0.7115	0.9412	0.2296	32	15	37	2
25.84419	0.2193	0.7308	0.9706	0.2398	33	14	38	1
24.241	0.2013	0.75	0.9706	0.2206	33	13	39	1
24.04282	0.1991	0.7692	0.9706	0.2014	33	12	40	1
22.96182	0.1877	0.7885	0.9706	0.1821	33	11	41	1
22.82823	0.1864	0.8077	0.9706	0.1629	33	10	42	1
22.1886	0.1799	0.8269	0.9706	0.1437	33	9	43	1
22.00162	0.178	0.8462	0.9706	0.1244	33	8	44	1
21.89138	0.1769	0.8654	0.9706	0.1052	33	7	45	1
21.82021	0.1762	0.8846	0.9706	0.086	33	6	46	1
21.8155	0.1762	0.9038	0.9706	0.0667	33	5	47	1
21.707	0.1751	0.9423	0.9706	0.0283	33	3	49	1
21.22106	0.1704	0.9615	0.9706	0.009	33	2	50	1
19.77987	0.1571	0.9615	1	0.0385	34	2	50	0
16.674	0.1312	0.9808	1	0.0192	34	1	51	0
12.51322	0.1023	1	1	0	34	0	52	0
12.51322	0.1023	1	1	0	34	0	52	0

Chapter 2 Table 3.

TBI+PTSD								
X	*Prob*	*1-Specificity*	*Sensitivity*	*Sens-(1-Spec)*	*True Pos*	*True Neg*	*False Pos*	*False Neg*
		0	0	0	0	52	0	13
76.5905	1	0	1	1*	13	52	0	0
12.51322	0	1	1	0	13	0	52	0
12.51322	0	1	1	0	13	0	52	0

Chapter 2 Table 4.

PTSD/TBI								
X	*Prob*	*1-Specificity*	*Sensitivity*	*Sens-(1-Spec)*	*True Pos*	*True Neg*	*False Pos*	*False Neg*
		0	0	0	0	21	0	34
1.296224	0.967	0	0.0294	0.0294	1	21	0	33
1.412363	0.9248	0	0.0588	0.0588	2	21	0	32
1.417704	0.922	0	0.0882	0.0882	3	21	0	31
1.426267	0.9172	0	0.1176	0.1176	4	21	0	30
1.492285	0.8712	0	0.1471	0.1471	5	21	0	29
1.494871	0.869	0	0.2059	0.2059	7	21	0	27
1.495408	0.8685	0	0.2353	0.2353	8	21	0	26
1.498919	0.8655	0.0476	0.2353	0.1877	8	20	1	26
1.500803	0.8639	0.0952	0.2353	0.1401	8	19	2	26
1.515669	0.8502	0.0952	0.2647	0.1695	9	19	2	25
1.539703	0.8259	0.0952	0.3235	0.2283	11	19	2	23
1.547916	0.8169	0.1429	0.3235	0.1807	11	18	3	23
1.553722	0.8103	0.1429	0.3529	0.2101	12	18	3	22
1.553745	0.8102	0.1905	0.3529	0.1625	12	17	4	22
1.579288	0.7791	0.1905	0.3824	0.1919	13	17	4	21
1.589118	0.7662	0.1905	0.4118	0.2213	14	17	4	20
1.593015	0.7609	0.1905	0.4412	0.2507	15	17	4	19
1.610618	0.7362	0.1905	0.4706	0.2801	16	17	4	18
1.62263	0.7183	0.1905	0.5	0.3095	17	17	4	17
1.625559	0.7139	0.2381	0.5	0.2619	17	16	5	17
1.653241	0.6698	0.2381	0.5294	0.2913	18	16	5	16
1.656371	0.6646	0.2381	0.5882	0.3501	20	16	5	14
1.6618	0.6554	0.2381	0.6176	0.3796	21	16	5	13
1.662663	0.654	0.2381	0.6471	0.409	22	16	5	12
1.664784	0.6504	0.2857	0.6471	0.3613	22	15	6	12
1.679373	0.6252	0.3333	0.6471	0.3137	22	14	7	12
1.686741	0.6122	0.3333	0.6765	0.3431	23	14	7	11
1.720267	0.5512	0.3333	0.7059	0.3725	24	14	7	10
1.721806	0.5484	0.3333	0.7353	0.402	25	14	7	9

1.72384	0.5446	0.381	0.7647	0.3838	26	13	8	8
1.732096	0.5292	0.381	0.7941	0.4132	27	13	8	7
1.735575	0.5227	0.381	0.8235	0.4426	28	13	8	6
1.749412	0.4969	0.381	0.8529	0.472*	29	13	8	5
1.76026	0.4766	0.4286	0.8529	0.4244	29	12	9	5
1.760624	0.4759	0.4762	0.8529	0.3768	29	11	10	5
1.77108	0.4564	0.4762	0.8824	0.4062	30	11	10	4
1.781068	0.438	0.4762	0.9118	0.4356	31	11	10	3
1.785739	0.4294	0.5238	0.9118	0.388	31	10	11	3
1.787461	0.4262	0.5714	0.9118	0.3403	31	9	12	3
1.789456	0.4226	0.5714	0.9412	0.3697	32	9	12	2
1.797938	0.4072	0.619	0.9412	0.3221	32	8	13	2
1.812061	0.3819	0.6667	0.9412	0.2745	32	7	14	2
1.823396	0.3621	0.6667	0.9706	0.3039	33	7	14	1
1.827528	0.355	0.6667	1	0.3333	34	7	14	0
1.844138	0.3271	0.7143	1	0.2857	34	6	15	0
1.847286	0.3219	0.7619	1	0.2381	34	5	16	0
1.851307	0.3154	0.8095	1	0.1905	34	4	17	0
1.85259	0.3133	0.8571	1	0.1429	34	3	18	0
1.878538	0.2731	0.9048	1	0.0952	34	2	19	0
1.878554	0.2731	0.9524	1	0.0476	34	1	20	0
1.888382	0.2588	1	1	0	34	0	21	0
1.888382	0.2588	1	1	0	34	0	21	0

Chapter 2 Table 5.

Chapter 3

Section 1

Plasma profiling of individual molecular species of PC, LPC, SM, PE, LPE, and PI in plasma of TBI, PTSD, and TBI+PTSD subjects

Chapter 3 Tables 1–6 show values of PL molecular species in plasma within each PL class identified by PCA to be significantly associated with the different diagnostic groups and the control group, as PCA also allows us to distinguish diagnostic groups, not only from control subjects but also from each other (TBI, compared with PTSD/TBI+PTSD and vice versa; see online supplementary material at www.liebertpub.com). For PC, multivariate PCA of the plasma data showed that component 1 explained 66% of the variance in the dataset and was associated with PTSD and TBI+PTSD ($p = 0.04$). Component 4 ($p = 0.007$), associated with TBI and TBI+PTSD, and component 5 ($p = 0.01$), associated with TBI+PTSD, each explained 3% of variance in the dataset. Supplementary Table 1 shows individual PC species and those that were specific to PCA components 1, 4, and 5 after post hoc adjustments. For LPC component 1 of the PCA, there was significance for TBI and TBI+PTSD, which explained 84% of the total variance in the dataset ($p < 0.001$). Supplementary Table 2 shows all individual LPC species that were identified to be significant by PCA in component 1. For PE, multivariate PCA of the plasma data showed that component 1 explained 79% of variance in the dataset and was associated with TBI+PTSD ($p = 0.05$). Component 2 explained 6% of variance in the dataset and was associated with TBI ($p = 0.03$). Supplementary Table 3 shows individual PE species, identified by components 1, 2, and 3. For LPE, component 1 of the PCA explained 62% of the total variance in the dataset ($F3, 351 = 8.2$, $p < 0.001$), and was significantly associated with TBI ($p = 0.007$), PTSD ($p = 0.01$), and TBI+PTSD ($p < 0.001$). Supplementary Table 4 shows individual LPE species that were specific to PCA component 1. For PI, component 1 explained 89% of the total variance ($p < 0.001$) and was associated with TBI ($p = 0.2$) and TBI+PTSD ($p < 0.001$). Supplementary Table 5 shows individual PI species in plasma with significant changes. For SM, component 2 explained 22% of the total variance ($p < 0.001$) and was significantly associated with TBI ($p = 0.001$), PTSD ($p = 0.05$), and TBI+PTSD ($p < 0.001$). Supplementary Table 6 shows individual significant SM species that were specifically significant with PCA component 2.

Table 1-6. Plasma levels of phosphatidylcholine (PC), lysophosphatidylcholines (LPC), phosphatidylethanolamine (PE), lysophosphatidylethanolamide (LPE), phosphatidylinositol (PI), and sphingomyelin (SM) molecular species quantified by liquid chromatography/mass spectrometry (LC/MS) analyses. Phospholipids presented in each table are those that were associated with principal component analysis component within each class that was significantly associated with traumatic brain injury (TBI), post-traumatic stress disorder (PTSD), and/or TBI+PTSD. Individual species for all lipid classes that are significant different, compared with controls, are indicated with * for $p < 0.05$. Values are represented in μM – standard error of the mean.

Chapter 3 Table 1. Individual PC Species

Phosphatidylcholine												
	Control			TBI			PTSD			TBI+PTSD		
ePC(32:0)	4.71	±	0.09	4.17	±	0.14*	4.26	±	0.13*	3.86	±	0.16*
PC(32:2)	5.34	±	0.20	4.53	±	0.29	4.85	±	0.27	3.42	±	0.27*#$
PC(32:0)	19.26	±	0.43	16.48	±	0.61*	17.59	±	0.52*	16.76	±	1.24*
ePC(34:3)	10.30	±	0.32	8.64	±	0.43	9.06	±	0.31*	7.95	±	0.27*
ePC(34:2)	12.03	±	0.42	10.35	±	0.50	10.44	±	0.41*	9.30	±	0.39*
ePC(34:1)	10.55	±	0.22	9.06	±	0.32*	9.56	±	0.27*	8.20	±	0.33*#$
ePC(34:0)	5.27	±	0.14	4.42	±	0.18*	4.69	±	0.16*	3.88	±	0.12*$
PC(34:3)	14.28	±	0.81	11.61	±	0.83	12.28	±	0.88	10.97	±	0.77*
PC(34:2)	630.71	±	19.69	488.47	±	22.00*	543.91	±	19.57*	465.35	±	16.40*
PC(34:1)	188.48	±	5.79	155.16	±	7.39*	169.31	±	6.03	143.97	±	6.63*
PC(34:0)	9.95	±	0.29	8.22	±	0.38	8.87	±	0.28	7.96	±	0.54*
ePC(36:5)	18.01	±	0.48	15.92	±	0.73	16.38	±	0.50	14.76	±	0.42*
ePC(36:4)	18.89	±	0.49	16.79	±	0.65	16.40	±	0.46*	14.82	±	0.46*#
ePC(36:3)	8.48	±	0.23	7.00	±	0.31*	7.39	±	0.23*	6.18	±	0.27*#$
ePC(36:2)	17.06	±	0.50	13.63	±	0.59*	14.70	±	0.54*	12.03	±	0.34*$
ePC(36:1)	9.02	±	0.29	7.10	±	0.38*	8.00	±	0.35	6.06	±	0.19*$
PC(36:4)	297.71	±	8.26	256.01	±	11.33	267.46	±	10.33	237.52	±	9.71*
PC(36:3)	164.83	±	4.84	124.12	±	5.91*	143.97	±	4.82*	122.40	±	8.08*$
PC(36:2)	269.71	±	7.45	206.73	±	9.73*	236.14	±	8.06*	192.75	±	10.27*$
PC(36:1)	42.69	±	1.68	29.62	±	2.10*	37.91	±	2.07	26.43	±	2.28*$
ePC(38:6)	8.80	±	0.21	7.79	±	0.30	7.87	±	0.23*	7.55	±	0.30*
ePC(38:5)	20.78	±	0.51	18.27	±	0.72	18.15	±	0.51*	16.64	±	0.51*#
ePC(38:4)	18.32	±	0.46	16.12	±	0.63	15.85	±	0.47*	14.47	±	0.45*#
ePC(38:3)	6.99	±	0.24	5.46	±	0.30*	5.99	±	0.27*	4.60	±	0.20*$
ePC(38:0)	2.94	±	0.08	2.54	±	0.11	2.60	±	0.10	2.31	±	0.10*
PC(38:6)	73.10	±	2.07	64.36	±	2.76	63.89	±	2.44*	65.01	±	3.11
PC(38:5)	77.24	±	2.08	65.05	±	2.73	67.54	±	2.32*	61.55	±	3.02*
PC(38:4)	178.52	±	4.95	151.08	±	7.04	160.03	±	5.90	140.15	±	7.48*
PC(38:3)	66.77	±	2.56	48.92	±	3.35*	57.62	±	2.95	41.73	±	3.89*$
PC(38:2)	6.42	±	0.39	4.30	±	0.37*	6.00	±	0.70	3.17	±	0.16*$
ePC(40:6)	5.59	±	0.15	4.91	±	0.20	4.77	±	0.17*	4.79	±	0.14*
ePC(40:5)	4.90	±	0.18	4.68	±	0.20	4.54	±	0.16*	4.61	±	0.14
PC(40:8)	4.19	±	0.12	3.49	±	0.16	3.73	±	0.14	2.99	±	0.09*#$
PC(40:7)	8.41	±	0.23	7.25	±	0.31	7.35	±	0.25*	7.18	±	0.31*
PC(40:6)	27.37	±	0.77	24.07	±	1.05	24.32	±	0.89*	24.20	±	1.31
PC(40:3)	1.70	±	0.08	1.33	±	0.09	1.48	±	0.09	1.16	±	0.04*

For PC, significant differences were observed to the control group (*p < 0.05), to the TBI group (#p < 0.05; PC[32:2], ePC[34:1], ePC[36:4], ePC[36:3], ePC[38:5], ePC[38:4], and PC[40:8]), and with the PTSD groups ($p < 0.05; PC[32:2], ePC[34:1], ePC[34:0] ePC[36:3], ePC[36:2], ePC[36:1], PC[36:3], PC[36:31], PC[36:1], ePC[38:3], PC[38:3], PC[38:2] and PC[40:8]. ePC, ether phosphatidylcholine.

Chapter 3 Table 2. LPC Significance

	Lysophosphatidylcholine			
	Control	**TBI**	**PTSD**	**TBI+PTSD**
LPC(0-16:0)	4.27 ± 0.12	3.46 ± 0.17*	3.73 ± 0.15*#	2.63 ± 0.10*#$
LPC(16:1)	8.35 ± 0.27	6.56 ± 0.35*	7.11 ± 0.30*#	5.19 ± 0.18*#$
LPC(16:0)	240.78 ± 7.40	188.18 ± 8.97*	196.57 ± 7.71*	159.45 ± 5.13*$
LPC(0-18:0)	7.76 ± 0.24	5.98 ± 0.27*	6.37 ± 0.25*#	5.10 ± 0.16*#$
LPC(18:3)	2.92 ± 0.09	2.28 ± 0.11*	2.65 ± 0.12	1.86 ± 0.06*#$
LPC(18:2)	138.39 ± 4.97	98.40 ± 5.21*	115.26 ± 4.26*	97.78 ± 4.29*
LPC(18:1)	57.04 ± 1.88	43.53 ± 2.39*	48.39 ± 1.88*	39.49 ± 1.45*$
LPC(18:0)	82.62 ± 2.75	63.09 ± 3.29*	67.59 ± 3.13*	54.83 ± 2.70*$
LPC(20:5)	2.26 ± 0.10	1.99 ± 0.10*	2.11 ± 0.13*	1.75 ± 0.06
LPC(20:4)	26.56 ± 0.95	21.58 ± 1.04*	23.03 ± 1.10*	20.21 ± 0.87
LPC(20:3)	9.30 ± 0.33	6.63 ± 0.38*	8.28 ± 0.42#	6.30 ± 0.36*$
LPC(20:2)	2.41 ± 0.08	1.81 ± 0.10*	2.00 ± 0.08*	1.61 ± 0.06*$
LPC(22:6)	4.92 ± 0.17	3.92 ± 0.20*	4.26 ± 0.20*	4.06 ± 0.16
LPC(22:5)	3.14 ± 0.11	2.42 ± 0.13*	2.71 ± 0.12*	2.22 ± 0.09*

For LPC, significance was observed in TBI+PTSD subjects for LPC species, LPC(0-16:0), LPC(16:1), LPC(0-18:0), and LPC(18:3). LPC(20:3) was significantly different between PTSD and TBI subjects. TBI+PTSD subjects showed significant differences, compared with PTSD ($p < 0.05), for lipid species LPC(0-16:0), LPC(16:1), LPC(16:0), LPC(0-18:0), LPC(18:3), LPC(18:1), LPC(18:0), and LPC(20:2).

Chapter 3 Table 3. PE Significance

Phosphatidylethanolamine												
	Control			TBI			PTSD			TBI+PTSD		
ePE(34:3)	4.66	±	0.18	3.43	±	0.21	4.08	±	0.15	2.88	±	0.17*#$
ePE(34:2)	4.57	±	0.25	3.21	±	0.30	3.69	±	0.24	3.33	±	0.75*
ePE(34:1)	2.81	±	0.04	2.61	±	0.06	2.65	±	0.05	2.48	±	0.08*$
ePE(34:0)	0.50	±	0.02	0.41	±	0.02*	0.44	±	0.02	0.34	±	0.01*#$
PE(34:4)	0.54	±	0.02	0.43	±	0.02*	0.47	±	0.02*	0.34	±	0.01*#$
PE(34:2)	5.35	±	0.24	3.70	±	0.26*	4.13	±	0.16*	3.64	±	0.45*#$
PE(34:1)	3.47	±	0.15	2.45	±	0.15*	2.92	±	0.12	2.40	±	0.29*$
ePE(36:5)	21.70	±	0.87	16.63	±	0.97*	18.65	±	0.72*	13.18	±	0.76*#$
ePE(36:4)	5.86	±	0.23	4.33	±	0.27*	5.04	±	0.17	3.70	±	0.23*#$
ePE(36:3)	10.63	±	0.56	7.10	±	0.60*	8.55	±	0.46	7.57	±	1.43*
ePE(36:2)	4.12	±	0.20	2.85	±	0.24*	3.30	±	0.19	2.83	±	0.51*
PE(36:5)	2.72	±	0.09	2.14	±	0.11*	2.26	±	0.07*	1.76	±	0.08*#$
PE(36:4)	6.39	±	0.23	4.84	±	0.31*	5.25	±	0.19*	4.29	±	0.45*$
PE(36:3)	5.83	±	0.27	3.71	±	0.27*	4.35	±	0.16*	4.06	±	0.44*
PE(36:2)	14.63	±	0.69	9.30	±	0.73*	10.87	±	0.45*	10.50	±	1.52*
ePE(38:6)	21.56	±	0.85	16.31	±	0.99*	18.26	±	0.68	14.06	±	0.90*$
ePE(38:5)	35.24	±	1.47	26.22	±	1.70*	29.22	±	1.15*	22.20	±	1.40*$
ePE(38:4)	5.13	±	0.21	3.62	±	0.21*	4.14	±	0.14*	3.33	±	0.29*$
ePE(38:3)	2.22	±	0.10	1.67	±	0.14	1.82	±	0.10	1.62	±	0.30*
PE(38:6)	4.74	±	0.19	3.83	±	0.19*	3.86	±	0.17*	3.45	±	0.43*
PE(38:5)	8.13	±	0.31	6.12	±	0.34*	6.64	±	0.26*	5.25	±	0.43*$
PE(38:4)	25.88	±	1.15	19.15	±	1.20*	21.21	±	0.92*	16.12	±	1.68*$
PE(38:3)	3.31	±	0.13	2.39	±	0.16*	2.73	±	0.12*	2.21	±	0.30*$
PE(38:1)	3.28	±	0.13	2.61	±	0.13	2.79	±	0.11	2.52	±	0.15*
PE(38:0)	8.04	±	0.32	6.03	±	0.33*	6.49	±	0.26*	5.95	±	0.47*
ePE(40:6)	8.61	±	0.37	6.45	±	0.40*	7.16	±	0.31	5.55	±	0.46*$
ePE(40:5)	8.20	±	0.42	6.34	±	0.45	6.88	±	0.34	5.06	±	0.48*$
ePE(40:4)	2.43	±	0.06	1.98	±	0.08*	2.15	±	0.06*	1.78	±	0.12*#$
ePE(40:3)	1.80	±	0.06	1.42	±	0.07*	1.54	±	0.06*	1.35	±	0.14*$
PE(40:6)	5.23	±	0.21	3.96	±	0.24*	4.29	±	0.21	4.08	±	0.73*
PE(40:5)	4.06	±	0.17	3.01	±	0.20*	3.32	±	0.15*	2.71	±	0.40*$
PE(42:7)	1.94	±	0.06	1.53	±	0.07*	1.72	±	0.06	1.25	±	0.05*#$
PE(42:6)	2.73	±	0.09	2.11	±	0.11*	2.36	±	0.08*	1.72	±	0.08*#$

For PE, ePE(34:3), ePE(34:0), PE(34:4), PE(34:2), ePE(36:5), ePE(36:4), PE(36:5), ePE(40:4), PE(42:7), and PE(42:6) were significantly different in TBI+PTSD, compared with TBI subjects (#$p<0.05$). Compared with the PTSD group, there were significant differences ($$p<0.05$) in TBI+PTSD subjects for lipid species ePE(34:3), ePE(34:1), ePE(34:0), PE(34:4), PE(34:2), PE(34:1), ePE(36:5), ePE(36:4), PE(36:5), PE(36:4), ePE(38:6), ePE(38:5), ePE(38:4), PE(38:5), PE(38:4), PE(38:3), ePE(40:6), ePE(40:5), ePE(40:4), ePE(40:3), PE(40:5), PE(42:7), and PE(42:6). ePE, ether phosphatidylethanolamine

Chapter 3 Table 4. LPE Significance

Lysophosphatidylethanolamine												
	Control			TBI			PTSD			TBI+PTSD		
LPE(o-16:1)	1.71	±	0.05	1.31	±	0.06*	1.43	±	0.05*	1.14	±	0.05*$
LPE(16:0)	3.44	±	0.16	2.90	±	0.18*	2.92	±	0.15*	2.68	±	0.12*
LPE(o-18:2)	0.82	±	0.05	0.66	±	0.05*	0.65	±	0.04*	0.71	±	0.07
LPE(o-18:1)	1.53	±	0.08	1.30	±	0.07*	1.31	±	0.06*	1.20	±	0.07*
LPE(18:3)	0.93	±	0.03	0.72	±	0.04*	0.80	±	0.03*	0.57	±	0.03*$
LPE(18:2)	13.89	±	0.61	9.01	±	0.62*	10.88	±	0.45*	9.32	±	0.69*
LPE(18:1)	6.11	±	0.30	3.88	±	0.27*	5.14	±	0.27*#	4.80	±	0.35
LPE(18:0)	4.35	±	0.24	3.48	±	0.23*	3.54	±	0.21*	3.27	±	0.21*
LPE(o-20:1)	4.29	±	0.08	4.16	±	0.11	4.11	±	0.10	3.66	±	0.14*#
LPE(20:4)	8.38	±	0.29	6.45	±	0.36*	7.05	±	0.34*	6.16	±	0.41*
LPE(22:6)	4.12	±	0.13	3.40	±	0.18*	3.50	±	0.17*	3.14	±	0.18*
LPE(22:5)	1.72	±	0.07	1.35	±	0.08*	1.46	±	0.07*	1.24	±	0.08*

For LPE, compared with the TBI group (#$p < 0.05$), LPE (0–20:1) in PTSD subjects and LPE(18:1) in TBI+PTSD subjects were significantly different. Differences in LPE(0–16:1) and LPE(18:3) were significant in TBI+PTSD subjects, compared with in PTSD subjects ($$p < 0.05$).

Chapter 3 Table 5. PI Significance

Phosphatidylinositol												
	Control			TBI			PTSD			TBI+PTSD		
PI(34:2)	9.79	±	0.46	6.49	±	0.53*	8.12	±	0.43*	5.50	±	0.21*$
PI(34:1)	7.89	±	0.36	5.23	±	0.45*	6.92	±	0.35	4.56	±	0.21*$
PI(34:0)	0.99	±	0.04	0.69	±	0.05*	0.84	±	0.05	0.59	±	0.03*$
PI(36:4)	11.79	±	0.50	8.62	±	0.70*	9.70	±	0.58*	7.13	±	0.43*$
PI(36:3)	8.80	±	0.43	5.51	±	0.44*	7.02	±	0.39*	5.03	±	0.34*$
PI(36:2)	29.69	±	1.36	18.97	±	1.45*	24.21	±	1.21*	16.96	±	0.65*$
PI(36:1)	8.98	±	0.40	5.85	±	0.51*	7.84	±	0.39	5.10	±	0.20*$
PI(36:0)	0.76	±	0.03	0.55	±	0.04*	0.66	±	0.03*	0.49	±	0.03*$
PI(37:4)	3.66	±	0.16	2.56	±	0.23	2.97	±	0.18*	2.21	±	0.39*#$
PI(38:6)	1.58	±	0.07	1.24	±	0.09	1.28	±	0.06*	1.06	±	0.05*
PI(38:5)	10.38	±	0.53	7.33	±	0.61	8.35	±	0.49	5.97	±	0.40*#$
PI(38:4)	124.11	±	5.62	90.86	±	6.85*	98.75	±	5.75*	76.49	±	3.30*$
PI(38:3)	19.96	±	0.95	13.15	±	1.00*	16.07	±	0.98*	12.05	±	0.99*$
PI(38:2)	2.34	±	0.11	1.51	±	0.11*	1.84	±	0.10*	1.39	±	0.10*$
PI(40:6)	3.62	±	0.14	2.83	±	0.20*	2.93	±	0.15*	2.49	±	0.12*
PI(40:5)	3.78	±	0.15	2.75	±	0.20*	3.10	±	0.16*	2.30	±	0.12*$
PI(40:4)	2.17	±	0.10	1.55	±	0.12*	1.70	±	0.09*	1.25	±	0.07*$

For PI, significant differences in TBI+PTSD subjects, compared with the PTSD group, were observed for all individual PI species, except PI(38:6) and PI(40:6), which were significant, compared to controls. PI(37:4) and PI(38:5) showed significant difference between TBI+PTSD and TBI subjects.

Chapter 3 Table 6. SM Significance

Sphingomyelin												
	Control			TBI			PTSD			TBI+PTSD		
SM(16:1)	36.73	±	1.22	29.58	±	1.63*	32.62	±	1.39*#	24.59	±	0.87*$
SM(16:0)	225.10	±	6.40	191.86	±	10.91*	195.94	±	6.06*	164.93	±	10.19*
DSM(16:0)	24.59	±	13.66	8.23	±	0.30*	9.89	±	0.43*#	7.69	±	0.27*$
SM(18:1)	22.68	±	0.77	18.71	±	0.98*	20.08	±	0.77*	16.47	±	0.85*
SM(18:0)	47.01	±	2.87	37.46	±	2.09*	40.76	±	1.61	33.42	±	2.25*
DSM(18:0)	14.52	±	4.27	7.52	±	0.46*	9.27	±	0.49*	6.84	±	0.64*
SM(22:0)	96.51	±	13.62	62.96	±	2.81*	76.43	±	3.38*#	57.56	±	4.81*$
DSM(22:0)	11.34	±	5.16	4.70	±	0.21*	5.92	±	0.24#	4.91	±	0.53
SM(24:1)	95.66	±	5.45	77.26	±	3.46*	82.07	±	3.87*	66.90	±	3.64*
SM(24:0)	44.96	±	7.10	32.24	±	1.65*	35.62	±	2.02	28.53	±	1.99*
DSM(24:0)	6.35	±	3.87	2.16	±	0.14*	2.22	±	0.13*	2.14	±	0.31

For SM, PTSD was significantly different from TBI (#$p < 0.05$) for individual species SM(16:1), DSM(16:0), SM(22:0), and DSM(22:0). TBI+PTSD showed significant differences, compared with PTSD subjects ($$p < 0.05$), for species SM(16:1), DSM(16:0), and SM(22:0).

Section 2

Chapter 4 Table 1. Model for TBI/Control

TBI/Control		
Positive if Greater Than or Equal Toa	Sensitivity	1 - Specificity
0	1	1
0.0003201	1	0.98
0.000728	1	0.959
0.0009059	1	0.939
0.0010088	1	0.918
0.0010308	1	0.898
0.0023843	1	0.878
0.0039458	1	0.857
0.0078302	1	0.837
0.0143355	1	0.816
0.018829	1	0.796
0.0216165	1	0.776
0.0229153	1	0.755
0.023284	1	0.735
0.0258332	1	0.714
0.0295138	1	0.694
0.0319376	1	0.673
0.0331461	1	0.653
0.0333842	1	0.633
0.0348726	1	0.612
0.0436122	1	0.592
0.0523973	1	0.571
0.0541673	1	0.551
0.0560511	1	0.531
0.0653177	1	0.51
0.0741049	1	0.49
0.0758687	1	0.469
0.0782528	1	0.449
0.0933067	1	0.429
0.1170664	1	0.408
0.1281328	1	0.388
0.1363335	1	0.367
0.1521556	1	0.347
0.1610514	0.952	0.347
0.1708775	0.952	0.327

0.2027056	0.905	0.327
0.2275636	0.905	0.306
0.2309259	0.857	0.306
0.2404489	0.81	0.306
0.2506981	0.81	0.286
0.2539207	0.81	0.265
0.264218	0.81	0.245
0.2731101	0.81	0.224
0.2886595	0.81	0.204
0.3091372	0.81	0.184
0.3154035	0.762	0.184
0.3595685	0.714	0.184
0.4050152	0.714	0.163
0.4128655	0.667	0.163
0.4314235	0.619	0.163
0.4493595	0.619	0.143
0.4616927	0.619	0.122
0.4858942	0.619	0.102
0.5278696	0.571	0.102
0.581578	0.524	0.102
0.6310951	0.524	0.082
0.6777047	0.524	0.061
0.7033585	0.524	0.041
0.7162581	0.476	0.041
0.7337488	0.429	0.041
0.7519871	0.429	0.02
0.7745748	0.429	0
0.7929965	0.381	0
0.8023577	0.333	0
0.8172826	0.286	0
0.8320203	0.238	0
0.8453654	0.19	0
0.8617333	0.143	0
0.9015343	0.095	0
0.9442715	0.048	0
1	0	0

PTSD/Control		
Positive if Greater Than or Equal Toa	Sensitivity	1 - Specificity
0	1	1
0.0794584	1	0.98
0.0898444	1	0.961
0.1168506	1	0.941
0.1425846	1	0.922
0.159953	1	0.902
0.1757084	1	0.882
0.1780795	1	0.863
0.1896353	1	0.843
0.2003984	1	0.824
0.2027674	1	0.804
0.21145	1	0.784
0.2217499	1	0.765
0.2243891	0.971	0.765
0.2252604	0.971	0.745
0.2303867	0.943	0.745
0.2369179	0.943	0.725
0.2394072	0.943	0.706
0.2399753	0.943	0.686
0.2404916	0.914	0.686
0.2409993	0.914	0.667
0.2453073	0.914	0.647
0.2626892	0.886	0.647
0.2855862	0.886	0.627
0.2956931	0.886	0.608
0.2990594	0.886	0.588
0.3032657	0.857	0.588
0.3135079	0.829	0.588
0.3232387	0.829	0.569
0.3260206	0.829	0.549
0.3319038	0.829	0.529
0.33647	0.829	0.51
0.3380085	0.829	0.49
0.3391003	0.8	0.49
0.3452105	0.771	0.49
0.3511471	0.771	0.471
0.3564882	0.743	0.471
0.3639014	0.743	0.451

0.3705299	0.714	0.451
0.3755812	0.714	0.431
0.3775026	0.686	0.431
0.3896955	0.686	0.412
0.4008059	0.686	0.392
0.4038276	0.657	0.392
0.4087202	0.629	0.392
0.4145029	0.629	0.373
0.4211236	0.6	0.373
0.4266404	0.6	0.353
0.4293396	0.571	0.353
0.4302646	0.543	0.353
0.4315886	0.543	0.333
0.4361004	0.543	0.314
0.4416025	0.543	0.294
0.4500837	0.543	0.275
0.4587867	0.514	0.275
0.4670146	0.514	0.255
0.4783581	0.514	0.235
0.4894846	0.514	0.216
0.4959783	0.486	0.216
0.502472	0.486	0.196
0.5105237	0.457	0.196
0.5176489	0.429	0.196
0.5305112	0.4	0.196
0.5385555	0.4	0.176
0.5423254	0.371	0.176
0.5489329	0.343	0.176
0.5647867	0.343	0.157
0.5786658	0.314	0.157
0.5843426	0.286	0.157
0.5888753	0.286	0.137
0.5924966	0.257	0.137
0.6002557	0.257	0.118
0.6055955	0.229	0.118
0.6069177	0.2	0.118
0.60739	0.2	0.098
0.6141478	0.171	0.098
0.6346784	0.171	0.078
0.6490615	0.171	0.059
0.6513235	0.171	0.039

0.6533999	0.143	0.039
0.6580722	0.114	0.039
0.6639494	0.086	0.039
0.6664918	0.086	0.02
0.6802207	0.057	0.02
0.7061777	0.029	0.02
0.7327367	0	0.02
1	0	0

TBI+PTSD/Control		
Positive if Greater Than or Equal Toa	Sensitivity	1 - Specificity
0	1	1
0.0001423	1	0.98
0.0003207	1	0.96
0.0004986	1	0.94
0.0006301	1	0.92
0.0008564	1	0.9
0.0010359	1	0.88
0.0010977	1	0.86
0.0015192	1	0.84
0.0019413	1	0.82
0.0027029	1	0.8
0.0044212	1	0.78
0.0079791	1	0.76
0.0105961	1	0.74
0.0109267	1	0.72
0.0127811	1	0.7
0.0143509	1	0.68
0.0160561	1	0.66
0.0215753	1	0.64
0.0263522	1	0.62
0.0281165	1	0.6
0.0297569	1	0.58
0.0309859	1	0.56
0.0337609	1	0.54
0.040487	1	0.52
0.0452289	1	0.5

0.0486148	0.909	0.5
0.0533577	0.909	0.48
0.0552101	0.909	0.46
0.0555933	0.909	0.44
0.0653878	0.909	0.42
0.0785234	0.909	0.4
0.089107	0.909	0.38
0.110792	0.909	0.36
0.1400743	0.818	0.36
0.1553989	0.818	0.34
0.1709385	0.818	0.32
0.1918754	0.818	0.3
0.2072906	0.818	0.28
0.2227882	0.818	0.26
0.2353613	0.818	0.24
0.2448624	0.818	0.22
0.2628385	0.818	0.2
0.2776911	0.818	0.18
0.2948441	0.727	0.18
0.3282904	0.636	0.18
0.3482269	0.636	0.16
0.3546619	0.636	0.14
0.3585686	0.636	0.12
0.3663632	0.636	0.1
0.3741141	0.545	0.1
0.3965565	0.545	0.08
0.4231576	0.455	0.08
0.4306512	0.455	0.06
0.4502956	0.364	0.06
0.4724377	0.273	0.06
0.5055574	0.273	0.04
0.5391119	0.273	0.02
0.5497214	0.273	0
0.64593	0.182	0
0.7600091	0.091	0
1	0	0

Chapter 4

Section 1

Chapter 4 table 1: Total plasma levels of PC, LPC, PE, LPE, PI and SM quantified by LC/MS analyses (µM± SEM). Individual molecular species of each class were quantified by LC/MS and summed after lipidomeDB analyses to generate total lipid levels. *denotes significant p values for all timepoints.

	Control			mTBI		
PC						
24h	1205.31	±	137.34	1140.15	±	38.82
3mo	1333.05	±	91.35	1071.09	±	63.22*
6mo	1409.66	±	142.51	1221.99	±	96.03
12mo	1463.37	±	53.10	1114.72	±	41.80*
24mo	519.72	±	49.31	408.83	±	31.44*
LPC						
24h	558.63	±	64.48	524.95	±	20.78
3mo	715.59	±	53.35	498.91	±	27.52*
6mo	761.90	±	91.89	598.23	±	61.14
12mo	660.39	±	38.03	571.40	±	27.72
24mo	585.54	±	79.58	432.14	±	38.16*
PE						
24h	52.07	±	6.61	59.62	±	3.61
3mo	60.53	±	4.39	45.07	±	2.59*
6mo	59.58	±	6.89	54.20	±	3.16
12mo	63.27	±	3.43	46.08	±	3.18*
24mo	24.73	±	2.18	21.56	±	0.62*
LPE						
24h	37.63	±	5.40	38.59	±	3.26
3mo	47.47	±	4.93	34.17	±	4.19*
6mo	41.28	±	5.70	32.81	±	4.15
12mo	39.26	±	3.17	33.98	±	2.38
24mo	33.59	±	3.92	25.19	±	1.47*
PI						
24h	191.61	±	7.95	174.70	±	11.66
3mo	231.73	±	19.07	170.28	±	19.17*
6mo	220.67	±	19.81	193.07	±	19.46
12mo	267.47	±	30.50	189.67	±	28.59*
24mo	114.43	±	12.51	85.04	±	7.83*
SM						
24h	46.17	±	4.95	51.04	±	2.16
3mo	44.14	±	4.96	39.71	±	2.54
6mo	58.24	±	4.69	47.98	±	4.39
12mo	50.10	±	4.52	42.20	±	1.63
24mo	17.97	±	2.34	15.47	±	0.89*

Chapter 4 table 2: Plasma levels of PC molecular species quantified by LC/MS analyses (µM± SEM).
Phospholipids presented in each table are those that were significantly associated with PCA component within each class that was significantly associated with mTBI at given timepoints.

Time-post injury	PC Species	Control			mTBI			mTBI/Control ratios
24h	ePC(38:6)	0.72	±	0.14	1.07	±	0.10	1.49
24h	PC(32:2)	0.94	±	0.13	0.66	±	0.10	0.70
24h	PC(34:3)	14.58	±	1.22	10.81	±	0.70	0.74
24h	PC(36:5)	7.31	±	0.64	5.64	±	0.28	0.77
24h	PC(40:4)	2.95	±	0.35	2.02	±	0.08	0.68
24h	PC(42:6)	0.94	±	0.17	0.59	±	0.06	0.63
3mo	ePC(34:2)	2.25	±	0.21	1.71	±	0.16	0.76
3mo	ePC(36:2)	5.93	±	0.41	4.82	±	0.41	0.81
3mo	ePC(36:4)	1.64	±	0.13	1.14	±	0.11	0.70
3mo	ePC(38:5)	1.89	±	0.14	1.38	±	0.13	0.73
3mo	PC(32:0)	11.21	±	0.77	9.07	±	0.87	0.81
3mo	PC(34:2)	359.17	±	23.82	288.48	±	19.96	0.80
3mo	PC(36:2)	160.36	±	12.30	126.30	±	9.67	0.79
3mo	PC(36:3)	76.53	±	5.40	62.04	±	4.19	0.81
3mo	PC(36:4)	187.98	±	11.27	149.62	±	7.79	0.80
3mo	PC(38:5)	48.32	±	3.22	38.90	±	1.84	0.81
6mo	PC(32:0)	12.51	±	1.35	9.44	±	0.79	0.75
12mo	ePC(34:1)	5.30	±	0.48	4.67	±	0.42	0.88
12mo	ePC(38:0)	3.98	±	0.21	2.74	±	0.29	0.69
12mo	ePC(38:4)	3.82	±	0.15	3.08	±	0.16	0.81
12mo	ePC(38:6)	1.10	±	0.07	0.77	±	0.12	0.70
12mo	ePC(40:6)	1.63	±	0.10	1.01	±	0.10	0.62
12mo	PC(32:0)	14.61	±	1.01	10.24	±	0.53	0.70
12mo	PC(34:0)	5.46	±	0.15	3.74	±	0.08	0.69
12mo	PC(34:3)	16.55	±	1.01	11.11	±	0.65	0.67
12mo	PC(36:5)	8.08	±	0.39	5.95	±	0.37	0.74
12mo	PC(40:5)	8.00	±	0.48	6.18	±	0.38	0.77
12mo	PC(40:7)	18.92	±	0.89	13.76	±	1.08	0.73
12mo	PC(34:1)	99.13	±	6.93	77.16	±	7.69	0.78
12mo	PC(34:2)	409.14	±	13.01	307.73	±	11.62	0.75
12mo	PC(36:2)	169.98	±	6.73	133.94	±	5.78	0.79
12mo	PC(36:3)	78.69	±	3.82	65.02	±	3.93	0.83
12mo	PC(36:4)	209.58	±	7.19	162.76	±	5.78	0.78
12mo	PC(38:4)	125.04	±	4.82	96.45	±	6.23	0.77
12mo	PC(38:5)	48.04	±	2.35	39.36	±	1.39	0.82
12mo	PC(38:6)	141.46	±	5.19	95.52	±	7.17	0.68
12mo	PC(40:6)	42.15	±	1.19	29.45	±	2.79	0.70
24mo	ePC(34:2)	0.14	±	0.05	0.47	±	0.06	3.24
24mo	ePC(36:1)	0.70	±	0.06	0.56	±	0.03	0.80
24mo	ePC(38:0)	1.48	±	0.17	0.86	±	0.12	0.58
24mo	PC(32:1)	2.75	±	0.33	1.44	±	0.24	0.52
24mo	PC(32:2)	0.50	±	0.09	0.23	±	0.05	0.46
24mo	PC(34:3)	5.12	±	0.64	3.41	±	0.40	0.67
24mo	PC(36:1)	9.19	±	0.78	6.41	±	0.69	0.70
24mo	PC(36:5)	2.94	±	0.31	1.85	±	0.24	0.63
24mo	PC(40:4)	0.88	±	0.11	0.63	±	0.04	0.72
24mo	PC(40:5)	3.76	±	0.38	2.94	±	0.25	0.78
24mo	PC(40:7)	9.78	±	0.98	6.34	±	0.62	0.65
24mo	PC(40:8)	2.75	±	0.28	1.83	±	0.22	0.67
24mo	PC(34:1)	48.24	±	4.69	31.75	±	5.15	0.66
24mo	PC(34:2)	122.13	±	14.06	96.28	±	7.33	0.79
24mo	PC(36:3)	36.53	±	4.61	25.47	±	2.11	0.70
24mo	PC(38:5)	23.96	±	2.14	16.74	±	1.73	0.70
24mo	PC(38:6)	42.90	±	3.67	34.05	±	3.14	0.79

Chapter 4 table 3: Plasma levels of LPC molecular species quantified by LC/MS analyses (µM± SEM).
Phospholipids presented in each table are those that were significantly associated with PCA component within each class that was significantly associated with mTBI at given timepoints.

Time-post injury	LPC species	Control			mTBI			mTBI/Control ratios
24h	LPC(16:1)	5.36	±	0.49	3.65	±	0.74	0.68
3mo	LPC(0-20:0)	1.89	±	0.17	1.07	±	0.24	0.57
3mo	LPC(16:1)	7.82	±	0.55	6.12	±	0.26	0.78
3mo	LPC(16:0)	239.39	±	13.87	167.44	±	8.63	0.70
3mo	LPC(18:0)	118.91	±	9.34	85.15	±	8.32	0.72
3mo	LPC(18:1)	71.42	±	6.57	50.26	±	2.87	0.70
3mo	LPC(18:2)	164.71	±	12.90	112.02	±	7.42	0.68
3mo	LPC(20:4)	65.65	±	6.83	42.45	±	2.23	0.65
3mo	LPC(22:6)	25.66	±	2.85	17.44	±	1.16	0.68
6mo	LPC(16:1)	8.43	±	1.09	6.16	±	0.75	0.73
6mo	LPC(22:6)	28.44	±	4.69	18.84	±	2.99	0.66
24mo	LPC(0-16:0)	1.44	±	0.26	0.78	±	0.15	0.54
24mo	LPC(16:1)	8.31	±	1.47	4.07	±	0.57	0.49
24mo	LPC(20:3)	9.59	±	1.42	5.95	±	0.98	0.62
24mo	LPC(16:0)	180.70	±	24.32	131.93	±	12.37	0.73
24mo	LPC(18:1)	84.68	±	12.89	52.47	±	8.10	0.62
24mo	LPC(18:2)	124.21	±	21.50	86.35	±	7.54	0.70

Chapter 4 table 4: Plasma levels of PE molecular species quantified by LC/MS analyses (µM± SEM).
Phospholipids presented in each table are those that were significantly associated with PCA component within each class that was significantly associated with mTBI at given timepoints.

Time-post injury	PE species	Control			mTBI			mTBI/Control ratios
24h	ePE(38:4)	1.12	±	0.17	1.65	±	0.09	1.48
24h	ePE(38:5)	1.60	±	0.20	2.29	±	0.12	1.43
24h	ePE(38:6)	1.79	±	0.20	2.64	±	0.18	1.48
24h	ePE(40:5)	0.75	±	0.10	1.04	±	0.07	1.40
24h	ePE(40:6)	2.31	±	0.30	3.49	±	0.27	1.51
24h	PE(38:0)	2.24	±	0.28	3.14	±	0.17	1.40
3mo	ePE(36:2)	1.18	±	0.11	0.83	±	0.05	0.70
3mo	ePE(36:4)	1.58	±	0.24	1.00	±	0.12	0.63
3mo	ePE(38:4)	1.73	±	0.16	1.20	±	0.04	0.70
3mo	ePE(38:5)	2.42	±	0.21	1.70	±	0.08	0.70
3mo	ePE(40:4)	2.23	±	0.17	1.76	±	0.10	0.79
3mo	ePE(40:5)	1.21	±	0.11	0.87	±	0.06	0.72
3mo	ePE(40:6)	2.99	±	0.27	2.29	±	0.11	0.77
3mo	PE(34:1)	0.26	±	0.06	0.15	±	0.04	0.56
3mo	PE(34:2)	1.64	±	0.14	1.05	±	0.14	0.64
3mo	PE(36:2)	3.35	±	0.31	2.31	±	0.27	0.69
3mo	PE(36:3)	1.59	±	0.12	1.08	±	0.13	0.68
3mo	PE(36:4)	2.90	±	0.19	2.02	±	0.17	0.70
3mo	PE(38:0)	3.21	±	0.30	2.19	±	0.10	0.68
3mo	PE(38:1)	0.66	±	0.07	0.45	±	0.04	0.68
3mo	PE(38:3)	0.45	±	0.04	0.31	±	0.03	0.69
3mo	PE(38:4)	5.10	±	0.34	3.73	±	0.19	0.73
3mo	PE(38:5)	3.69	±	0.33	2.86	±	0.20	0.77
3mo	PE(38:6)	6.00	±	0.45	4.50	±	0.23	0.75
3mo	PE(40:5)	1.01	±	0.12	0.64	±	0.08	0.63
3mo	PE(40:6)	1.89	±	0.19	1.35	±	0.07	0.72
3mo	PE(42:6)	0.38	±	0.03	0.23	±	0.03	0.60
12mo	ePE(36:2)	1.28	±	0.09	0.93	±	0.10	0.72
12mo	ePE(38:4)	2.07	±	0.11	1.32	±	0.13	0.64
12mo	ePE(38:5)	2.70	±	0.15	2.02	±	0.17	0.75

12mo	ePE(38:6)	2.23	±	0.10	1.58	±	0.13	0.71
12mo	ePE(40:4)	2.46	±	0.21	1.86	±	0.21	0.75
12mo	ePE(40:5)	1.39	±	0.10	1.00	±	0.10	0.72
12mo	ePE(40:6)	3.21	±	0.15	2.34	±	0.22	0.73
12mo	PE(34:2)	1.70	±	0.11	1.25	±	0.15	0.74
12mo	PE(36:2)	3.66	±	0.26	2.85	±	0.33	0.78
12mo	PE(36:4)	2.73	±	0.20	1.94	±	0.13	0.71
12mo	PE(38:0)	3.49	±	0.14	2.59	±	0.20	0.74
12mo	PE(38:1)	0.84	±	0.07	0.49	±	0.05	0.58
12mo	PE(38:3)	0.45	±	0.03	0.33	±	0.03	0.74
12mo	PE(38:4)	5.50	±	0.36	3.65	±	0.32	0.66
12mo	PE(38:5)	3.58	±	0.27	2.51	±	0.21	0.70
12mo	PE(38:6)	5.96	±	0.42	3.35	±	0.41	0.56
12mo	PE(40:6)	2.03	±	0.13	1.24	±	0.13	0.61
12mo	PE(40:7)	1.62	±	0.16	0.90	±	0.11	0.56
12mo	PE(42:5)	0.33	±	0.05	0.22	±	0.02	0.65
12mo	PE(42:6)	0.46	±	0.03	0.33	±	0.03	0.71
24mo	ePE(40:4)	1.06	±	0.11	0.87	±	0.06	0.81
24mo	ePE(40:5)	0.44	±	0.07	0.32	±	0.01	0.72
24mo	PE(38:5)	1.36	±	0.14	1.06	±	0.05	0.79
24mo	PE(38:6)	1.61	±	0.13	1.21	±	0.07	0.75

Chapter 4 table 5: Plasma levels of LPE molecular species quantified by LC/MS analyses (µM± SEM).
Phospholipids presented in each table are those that were significantly associated with PCA component within each class that was significantly associated with mTBI at given timepoints.

Time-post injury	LPE species	Control			mTBI			mTBI/Control ratios
3mo	LPE(16:0)	8.83	±	0.70	4.79	±	0.61	0.54
3mo	LPE(18:1)	3.05	±	0.37	2.01	±	0.29	0.66
6mo	LPE(16:0)	7.64	±	0.87	4.28	±	1.05	0.56
12mo	LPE(22:6)	6.95	±	0.51	4.36	±	0.34	0.63
24mo	LPE(18:1)	2.45	±	0.39	1.82	±	0.20	0.74
24mo	LPE(18:2)	4.57	±	0.81	2.76	±	0.26	0.60
24mo	LPE(18:3)	0.09	±	0.03	0.03	±	0.02	0.39
24mo	LPE(20:4)	6.19	±	0.80	3.62	±	0.33	0.59
24mo	LPE(22:6)	5.57	±	0.67	3.15	±	0.27	0.57

Chapter 4 table 6: Plasma levels of PI molecular species quantified by LC/MS analyses (µM± SEM).
Phospholipids presented in each table are those that were significantly associated with PCA component within each class that was significantly associated with mTBI at given timepoints.

Time-post injury	PI species	Control			mTBI			mTBI/Control ratios
24h	PI(34:2)	8.12	±	0.57	5.83	±	0.61	0.72
24h	PI(36:3)	6.15	±	0.38	4.82	±	0.34	0.78
24h	PI(36:4)	18.97	±	1.05	13.18	±	1.06	0.69
3mo	PI(36:3)	7.18	±	0.52	5.22	±	0.51	0.73
3mo	PI(38:4)	136.92	±	12.69	98.62	±	13.21	0.72
3mo	PI(38:5)	12.50	±	1.24	8.72	±	0.84	0.70
3mo	PI(37:4)	1.52	±	0.08	1.08	±	0.09	0.71
3mo	PI(38:2)	1.00	±	0.07	0.69	±	0.08	0.69
3mo	PI(38:6)	1.71	±	0.10	1.27	±	0.12	0.74
3mo	PI(40:7)	0.50	±	0.02	0.38	±	0.03	0.75

3mo	PI(42:3)	2.94	±	0.39	1.98	±	0.27	0.67
12mo	PI(34:2)	8.99	±	1.04	6.07	±	0.63	0.68
12mo	PI(36:2)	17.30	±	1.84	11.58	±	0.98	0.67
12mo	PI(36:3)	7.93	±	0.96	5.54	±	0.61	0.70
12mo	PI(38:3)	18.97	±	2.55	12.49	±	2.31	0.66
12mo	PI(38:4)	160.53	±	18.35	110.95	±	18.61	0.69
12mo	PI(38:2)	1.36	±	0.12	0.92	±	0.13	0.68
12mo	PI(40:5)	2.63	±	0.34	1.90	±	0.26	0.72
12mo	PI(40:6)	4.03	±	0.43	2.97	±	0.31	0.74
24mo	PI(36:4)	8.17	±	0.89	5.78	±	0.47	0.71
24mo	PI(38:3)	8.65	±	1.25	5.11	±	0.62	0.59
24mo	PI(38:4)	64.19	±	6.38	48.38	±	5.19	0.75
24mo	PI(38:5)	8.89	±	1.08	5.91	±	0.63	0.66
24mo	PI(36:6)	1.86	±	0.22	1.20	±	0.18	0.64
24mo	PI(38:2)	0.58	±	0.11	0.39	±	0.04	0.67
24mo	PI(38:6)	0.86	±	0.11	0.66	±	0.07	0.76
24mo	PI(40:4)	1.46	±	0.20	0.75	±	0.07	0.51
24mo	PI(40:5)	1.46	±	0.22	1.04	±	0.10	0.71

Chapter 4 table 7: Plasma levels of SM molecular species quantified by LC/MS analyses (µM± SEM).
Phospholipids presented in each table are those that were significantly associated with PCA component within each class that was significantly associated with mTBI at given timepoints

Time-post injury	SM species	Control			mTBI			mTBI/Control ratios
6mo	DSM(16:0)	0.65	±	0.06	0.36	±	0.02	0.56
6mo	DSM(18:0)	0.79	±	0.09	0.37	±	0.08	0.46
6mo	SM(18:0)	5.59	±	0.64	0.64	±	0.34	0.12
24mo	DSM(16:0)	0.19	±	0.05	0.25	±	0.02	1.36
24mo	DSM(18:0)	0.15	±	0.04	0.20	±	0.01	1.36
24mo	SM(18:0)	1.84	±	0.24	1.25	±	0.08	0.68
24mo	SM(22:1)	2.76	±	0.35	2.15	±	0.12	0.78
24mo	SM(24:0)	2.97	±	0.45	1.79	±	0.17	0.60

Chapter 5

Section 1 – Neurocognitive Measures

Visual Analog Scale (VAS)

INFORMATION POINT:

Visual Analogue Scale (VAS)

A Visual Analogue Scale (VAS) is a measurement instrument that tries to measure a characteristic or attitude that is believed to range across a continuum of values and cannot easily be directly measured. For example, the amount of pain that a patient feels ranges across a continuum from none to an extreme amount of pain. From the patient's perspective this spectrum appears continuous – their pain does not take discrete jumps, as a categorization of none, mild, moderate and severe would suggest. It was to capture this idea of an underlying continuum that the VAS was devised.

Operationally a VAS is usually a horizontal line, 100 mm in length, anchored by word descriptors at each end, as illustrated in Fig. 1. The patient marks on the line the point that they feel represents their perception of their current state. The VAS score is determined by measuring in millimetres from the left hand end of the line to the point that the patient marks.

How severe is your pain today? Place a vertical mark on the line below to indicate how bad you feel your pain is today.

No pain |_____| Very severe pain

Figure 1 Effects of the interpersonal, technical and communication skills of the nurse on the effectiveness of treatment.

There are many other ways in which VAS have been presented, including vertical lines and lines with extra descriptors. Wewers & Lowe (1990) provide an informative discussion of the benefits and shortcomings of different styles of VAS.

As such an assessment is clearly highly subjective, these scales are of most value when looking at change within individuals, and are of less value for comparing across a group of individuals at one time point. It could be argued that a VAS is trying to produce interval/ratio data out of subjective values that are at best ordinal. Thus, some caution is required in handling such data. Many researchers prefer to use a method of analysis that is based on the rank ordering of scores rather than their exact values, to avoid reading too much into the precise VAS score.

Further reading

Wewers M.E. & Lowe N.K. (1990) A critical review of visual analogue scales in the measurement of clinical phenomena. ***Research in Nursing and Health*** **13**, 227–236.

NICOLA CRICHTON

Multi-dimensional Fatigue Inventory (MFI-20)

Purpose The MFI is a 20-item scale designed to evaluate five dimensions of fatigue: general fatigue, physical fatigue, reduced motivation, reduced activity, and mental fatigue. By limiting the length of the questionnaire, developers hoped to accommodate those individuals who might find larger measures especially tiring while still obtaining enough detailed information to examine multiple facets of fatigue.

Population for Testing The scale has been validated in a variety of participant populations, including cancer patients (mean age of 61 years), army recruits (mean age of 21 years), psychology students (mean age of 24 years), and individuals participating in a study of chronic fatigue syndrome (mean age of 39 years).

Administration The MFI is a self-report, pencil-and-paper measure requiring between 5 and 10 min for completion.

Reliability and Validity In an initial psychometric evaluation [1], developers reported an internal consistency ranging from .53 to .93. The scale was also found to be sensitive to differences between the participant groups.

Obtaining a Copy An example of the questionnaire format is included in the original article published by developers [1].

For a complete copy, direct correspondence to:
E.M.A. Smets
Academic Medical Centre,
University of Amsterdam
Department of Medical Psychology
Amsterdam, the Netherlands

Scoring Respondents use a scale ranging from 1 to 7 to indicate how aptly certain statements regarding fatigue represent their experiences. Several positively phrased items are reverse-scored. Higher total scores correspond with more acute levels of fatigue.

MFI® MULTIDIMENSIONAL FATIGUE INVENTORY

® E. Smets, B.Garssen, B. Bonke.

Instructions:

By means of the following statements we would like to get an idea of how you have been feeling **lately**.
There is, for example, the statement:

"I FEEL RELAXED"

If you think that this is **entirely true**, that indeed you have been feeling relaxed lately, please, place an **X** in the extreme left box; like this:

yes, that is true ☒1 ❑2 ❑3 ❑4 ❑5 no, that is not true

The more you **disagree** with the statement, the more you can place an **X** in the direction of "no, that is not true". Please do not miss out a statement and place only one **X** in a box for each statement.

1	I feel fit.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
2	Physically, I feel only able to do a little.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
3	I feel very active.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
4	I feel like doing all sorts of nice things.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
5	I feel tired.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
6	I think I do a lot in a day.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
7	When I am doing something, I can keep my thoughts on it.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
8	Physically I can take on a lot.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
9	I dread having to do things.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
10	I think I do very little in a day.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
11	I can concentrate well.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
12	I am rested.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
13	It takes a lot of effort to concentrate on things.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
14	Physically I feel I am in a bad condition.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
15	I have a lot of plans.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
16	I tire easily.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
17	I get little done.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
18	I don't feel like doing anything.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
19	My thoughts easily wander.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true
20	Physically I feel I am in an excellent condition.	yes, that is true	❑1	❑2	❑3	❑4	❑5	no, that is not true

36-Item Short Form Survey Instrument (SF-36)

RAND 36-Item Health Survey 1.0 Questionnaire Items

Choose one option for each questionnaire item.

1. In general, would you say your health is:

- ○ 1 - Excellent
- ○ 2 - Very good
- ○ 3 - Good
- ○ 4 - Fair
- ○ 5 - Poor

---

2. **Compared to one year ago**, how would you rate your health in general **now**?

- ○ 1 - Much better now than one year ago
- ○ 2 - Somewhat better now than one year ago
- ○ 3 - About the same
- ○ 4 - Somewhat worse now than one year ago
- ○ 5 - Much worse now than one year ago

The following items are about activities you might do during a typical day. Does **your health now limit you** in these activities? If so, how much?

	Yes, limited a lot	Yes, limited a little	No, not limited at all
3. **Vigorous activities**, such as running, lifting heavy objects, participating in strenuous sports	○ 1	○ 2	○ 3
4. **Moderate activities**, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf	○ 1	○ 2	○ 3
5. Lifting or carrying groceries	○ 1	○ 2	○ 3
6. Climbing **several** flights of stairs	○ 1	○ 2	○ 3
7. Climbing **one** flight of stairs	○ 1	○ 2	○ 3
8. Bending, kneeling, or stooping	○ 1	○ 2	○ 3
9. Walking **more than a mile**	○ 1	○ 2	○ 3
10. Walking **several blocks**	○ 1	○ 2	○ 3
11. Walking **one block**	○ 1	○ 2	○ 3
12. Bathing or dressing yourself	○ 1	○ 2	○ 3

During the **past 4 weeks**, have you had any of the following problems with your work or other regular daily activities **as a result of your physical health**?

	Yes	No
13. Cut down the **amount of time** you spent on work or other activities	○ 1	○ 2
14. **Accomplished less** than you would like	○ 1	○ 2
15. Were limited in the **kind** of work or other activities	○ 1	○ 2
16. Had **difficulty** performing the work or other activities (for example, it took extra effort)	○ 1	○ 2

During the **past 4 weeks**, have you had any of the following problems with your work or other regular daily activities **as a result of any emotional problems** (such as feeling depressed or anxious)?

	Yes	No
17. Cut down the **amount of time** you spent on work or other activities	○ 1	○ 2
18. **Accomplished less** than you would like	○ 1	○ 2
19. Didn't do work or other activities as **carefully** as usual	○ 1	○ 2

---

20. During the **past 4 weeks**, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbors, or groups?

- ○ 1 - Not at all
- ○ 2 - Slightly
- ○ 3 - Moderately
- ○ 4 - Quite a bit
- ○ 5 - Extremely

---

21. How much **bodily** pain have you had during the **past 4 weeks**?

- 1 - None
- 2 - Very mild
- 3 - Mild
- 4 - Moderate
- 5 - Severe
- 6 - Very severe

22. During the **past 4 weeks**, how much did **pain** interfere with your normal work (including both work outside the home and housework)?

- 1 - Not at all
- 2 - A little bit
- 3 - Moderately
- 4 - Quite a bit
- 5 - Extremely

These questions are about how you feel and how things have been with you **during the past 4 weeks**. For each question, please give the one answer that comes closest to the way you have been feeling.

How much of the time during the **past 4 weeks**...

	All of the time	Most of the time	A good bit of the time	Some of the time	A little of the time	None of the time
23. Did you feel full of pep?	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6
24. Have you been a very nervous person?	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6
25. Have you felt so down in the dumps that nothing could cheer you up?	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6
26. Have you felt calm and peaceful?	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6
27. Did you have a lot of energy?	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6
28. Have you felt downhearted and blue?	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6
29. Did you feel worn out?	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6
30. Have you been a happy person?	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6
31. Did you feel tired?	○ 1	○ 2	○ 3	○ 4	○ 5	○ 6

32. During the **past 4 weeks**, how much of the time has **your physical health or emotional problems** interfered with your social activities (like visiting with friends, relatives, etc.)?

- ○ 1 - All of the time
- ○ 2 - Most of the time
- ○ 3 - Some of the time
- ○ 4 - A little of the time
- ○ 5 - None of the time

How TRUE or FALSE is **each** of the following statements for you.

	Definitely true	Mostly true	Don't know	Mostly false	Definitely false
33. I seem to get sick a little easier than other people	1	2	3	4	5
34. I am as healthy as anybody I know	1	2	3	4	5
35. I expect my health to get worse	1	2	3	4	5
36. My health is excellent	1	2	3	4	5

Profile of Mood States (POMS)

Directions: Describe HOW YOU FEEL RIGHT NOW
by circling the most appropriate number after each of the words listed below:

FEELING	Not at all	A little	Moderate	Quite a bit	Extremely
1. Friendly	1	2	3	4	5
2. Tense	1	2	3	4	5
3. Angry	1	2	3	4	5
4. Worn Out	1	2	3	4	5
5. Unhappy	1	2	3	4	5
6. Clear-headed	1	2	3	4	5
7. Lively	1	2	3	4	5
8. Confused	1	2	3	4	5
9. Sorry for things done	1	2	3	4	5
10. Shaky	1	2	3	4	5
11. Listless	1	2	3	4	5
12. Peeved	1	2	3	4	5
13. Considerate	1	2	3	4	5
14. Sad	1	2	3	4	5
15. Active	1	2	3	4	5
16. On edge	1	2	3	4	5
17. Grouchy	1	2	3	4	5
18. Blue	1	2	3	4	5
19. Energetic	1	2	3	4	5
20. Panicky	1	2	3	4	5
21. Hopeless	1	2	3	4	5
22. Relaxed	1	2	3	4	5
23. Unworthy	1	2	3	4	5
24. Spiteful	1	2	3	4	5
25. Sympathetic	1	2	3	4	5
26. Uneasy	1	2	3	4	5
27. Restless	1	2	3	4	5
28. Unable to	1	2	3	4	5
29. Fatigued	1	2	3	4	5

30. Helpful	1	2	3	4	5
31. Annoyed	1	2	3	4	5
32. Discouraged	1	2	3	4	5
33. Resentful	1	2	3	4	5
34. Nervous	1	2	3	4	5
35. Lonely	1	2	3	4	5
36. Miserable	1	2	3	4	5
37. Muddled	1	2	3	4	5
38. Cheerful	1	2	3	4	5
39. Bitter	1	2	3	4	5
40. Exhausted	1	2	3	4	5
41. Anxious	1	2	3	4	5
42. Ready to fight	1	2	3	4	5
43. Good-natured	1	2	3	4	5
44. Gloomy	1	2	3	4	5
45. Desperate	1	2	3	4	5
46. Sluggish	1	2	3	4	5
47. Rebellious	1	2	3	4	5
48. Helpless	1	2	3	4	5
49. Weary	1	2	3	4	5
50. Bewildered	1	2	3	4	5
51. Alert	1	2	3	4	5
52. Deceived	1	2	3	4	5
53. Furious	1	2	3	4	5
54. Effacious	1	2	3	4	5
55. Trusting	1	2	3	4	5
56. Full of pep	1	2	3	4	5
57. Bad-tempered	1	2	3	4	5
58. Worthless	1	2	3	4	5
59. Forgetful	1	2	3	4	5
60. Carefree	1	2	3	4	5
61. Terrified	1	2	3	4	5
62. Guilty	1	2	3	4	5
63. Vigorous	1	2	3	4	5
64. Uncertain about things	1	2	3	4	5
65. Bushed	1	2	3	4	5

Section 2 – Supplemental tables

Chapter 5 table 1: Total plasma levels for mice and rat model of PC, LPC, PE, LPE, PI and SM quantified by LC/MS analyses (Percentage of control± SEM). Individual molecular species of each class were quantified by LC/MS and summed after lipidomeDB analyses to generate total lipid levels. *denotes significant p values for p<0.05.

	Mouse model						Rat model					
	Control			PB+PER exposed			Control			PB+PER+DEET+Stress		
total PC	100	±	3.35	101.71	±	5.92	100	±	4.84	230.44	±	11.94*
total LPC	100	±	2.92	121.48	±	4.09*	100	±	6.04	160.84	±	6.91*
total PE	100	±	4.73	97.13	±	5.43	100	±	3.38	142.82	±	5.63*
total LPE	100	±	2.77	112.12	±	5.57*	100	±	2.15	94.87	±	2.26
total PI	100	±	2.60	106.33	±	3.19	100	±	4.96	239.53	±	10.84*
total SM	100	±	2.58	104.05	±	2.94	100	±	5.66	184.15	±	7.56

Chapter 5 table 2: Plasma levels for mice and rat model of SFA, MUFA and PUFA containing PC, LPC, PE, LPE and PI species quantified by LC/MS analyses (Percentage of control± SEM). *denotes significant p values for p<0.05.

		Mouse model						Rat model					
		Control			PB+PER			Control			PB+PER+DEET+Stress		
PC	*SFA*	100	±	4.43	99.45	±	7.31	100	±	2.18	219.45	±	9.63*
	MUFA	100	±	5.05	121.45	±	12.81	100	±	4.02	222.58	±	12.22*
	PUFA	100	±	3.95	97.44	±	6.08	100	±	5.99	236.86	±	15.09*
LPC	*SFA*	100	±	3.12	115.89	±	3.55*	100	±	6.39	147.57	±	6.63*
	MUFA	100	±	3.34	135.39	±	10.24*	100	±	4.64	172.86	±	6.45*
	PUFA	100	±	4.14	127.21	±	4.58*	100	±	6.05	184.28	±	8.65*
PE	*SFA*	100	±	5.80	94.04	±	4.47*	100	±	5.88	161.83	±	7.62*
	MUFA	100	±	3.25	103.91	±	7.26	100	±	3.49	128.50	±	3.55*
	PUFA	100	±	5.01	95.75	±	5.20*	100	±	3.38	143.03	±	6.55*
LPE	*SFA*	100	±	4.78	105.08	±	4.75	100	±	11.16	50.42	±	20.60*
	MUFA	100	±	4.26	105.10	±	6.87	100	±	1.74	96.11	±	1.90
	PUFA	100	±	3.20	116.46	±	7.08	100	±	11.83	105.98	±	19.89
PI	*SFA*	100	±	13.77	113.27	±	14.56	100	±	12.32	129.48	±	6.89*
	MUFA	100	±	4.99	113.60	±	5.72	100	±	12.37	36.37	±	1.78*
	PUFA	100	±	2.47	105.84	±	3.45	100	±	5.31	241.51	±	11.38*

Chapter 5 table 3: Plasma levels for mice and rat model of ether containing PC, LPC, PE and LPE species quantified by LC/MS analyses (Percentage of control± SEM) *denotes significant p values for p<0.05.

	Mouse model						Rat model					
	Control			PB+PER			Control			PB+PER+DEET+Stress		
ePC	100	4.98	±	96.80	±	8.90	100	±	5.37	226.37	±	11.92*
eLPC	100	5.14	±	100.61	±	6.86	100	±	4.36	144.96	±	4.04*
ePE	100	4.05	±	96.56	±	4.26	100	±	7.19	129.74	±	4.91*
eLPE	100	4.64	±	104.87	±	7.42	100	±	1.74	96.11	±	1.90

Chapter 5 table 4: Plasma levels for mice and rat model of AA, DHA and AAtoDHA ratios containing PC, LPC, PE and LPE species quantified by LC/MS analyses (Percentage of control± SEM). No DHA containing LPE species were identified for the rat model, therefore ratio for AAtoDHA containing LPE species could not be determined.*denotes significant p values for p<0.05.

		Mouse model						Rat model					
		Control			PB+PER			Control			PB+PER+DEET+Stress		
PC	*DHA*	100	±	4.53	89.36	±	5.73	100	±	6.75	274.99	±	26.61*
	AA	100	±	4.11	97.15	±	5.40	100	±	6.04	225.09	±	12.75*
LPC	*DHA*	100	±	5.16	115.56	±	7.92	100	±	11.04	230.95	±	23.45*
	AA	100	±	3.88	128.34	±	4.59*	100	±	6.77	180.23	±	11.26*
PE	*DHA*	100	±	5.65	89.27	±	3.99	100	±	4.97	198.35	±	14.97*
	AA	100	±	5.17	94.32	±	5.28	100	±	5.66	160.76	±	11.38*
LPE	*DHA*	100	±	2.89	116.71	±	8.11				N/A		
	AA	100	±	3.47	119.89	±	8.60*	100	±	31.43	144.36	±	36.57
PI	*DHA*	100	±	2.57	102.78	±	3.01	100	±	7.60	286.61	±	28.20*
	AA	100	±	2.19	102.60	±	3.02	100	±	6.16	245.68	±	11.53*